

School of Biomedical Sciences

**Investigations of Inflammatory Mechanisms Contributing to the
Pathogenesis of SLE in a Western Australian Population**

Audrey Margery-Muir

**This thesis is presented for the Degree of
Doctor of Philosophy
of
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Author's Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics: The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number # HR 202/2013 and from the Sir Charles Gairdner Group & Royal Perth Hospital Human Research Ethics Committees, Approval Number # HREC 2013 -174.

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Preface

This project had its genesis in the hypothesis that aberrant apoptotic processing of cellular debris generated inflammatory mechanisms that manifested as SLE. It was proposed therefore that routine laboratory investigations used in the diagnosis and management of SLE should be interpreted in this light, as well as other tests that may reflect the above hypothesis.

The main features of the “defective apoptosis” hypothesis as one of the important general mechanisms underlying SLE postulates the following sub-mechanisms. In normal physiological conditions cells die by way of apoptosis, and new cells are generated, after which the cell dies and is removed by scavenger cells such as macrophages and dendritic cells. If the cells are not removed in timely manner, they might undergo secondary necrosis, and as they break down, they release their contents, such as nucleic acids like DNA. The released materials generate an immune response including the activation of complement molecules, dendritic cells, T cells and B cells to produce large amounts of specific autoantibodies. Alternatively, a deficiency in complement molecules, such as C1q, or of the enzyme DNASE I, leads to an accumulation of apoptotic cells and debris that are taken up by dendritic cells which express co-stimulatory molecules CD40 and CD40LG. This results in inflammatory cytokines (i.e. IL-6) production, which, in the presence of TGF- β activates Th17 cells. This process causes auto-reactive antibody-producing B cells to form more immune complexes (IC). Environmental triggers, such as viral infection (in particular Epstein Barr virus) activate and are taken up by plasmacytoid DCs that specifically respond to viral infections by interferon alpha (INF- α) secretions, which, in association with the factors described above, leads to increased antibody production and formation of more immune complexes. The end-result of this aberrant activation is the tissue and organ damage seen in SLE patients.

A literature review is presented in Chapter 1 that takes an overview of the genetic factors that contribute to the pathogenesis of SLE; it questions the underlying rationale for the observed female to male gender imbalance characteristic of this important systemic autoimmune disease. A shortened version of the literature

review was published in Autoimmunity Reviews (2017). When topics pertaining to the laboratory diagnosis of SLE that were investigated further during this project they will be specifically identified at that time.

Three of the four experimental chapters in this thesis have been published prior to submission. A compilation of the methods used, with some additional explanatory notes, has been included in chapter 2 for the convenience of the readers and/or examiners and to avoid unnecessary repetition between chapters.

Chapter 3 of the thesis reports on studies of some of the cellular mechanisms underlying SLE inflammatory pathways. As described in the above paragraphs these relate to cell mediated acute and chronic inflammation and tissue damage resulting from defective clearance. These include studies of myeloid dendritic cells types I and II (mDC1 & mDC2), plasmacytoid dendritic cells (pDCs), regulatory T cells (Treg), gamma delta T cells ($\gamma\delta$ T cells) and helper T cells 17 (Th17 cells). In addition, a study of the inflammatory cytokine profile that include Interleukin 1 beta (IL1 β), interferon alpha (IFN α), interferon gamma (IFN γ), tumour necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1/ CCL2), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12p70), interleukin 17A (IL-17), interleukin 18 (IL-18), interleukin 23 (IL-23) and interleukin 33 (IL-33) concentrations in serum of SLE patients and healthy controls was undertaken.

Activation of the classical complement pathway is well described and known to be a potent contributor to inflammatory processes. Given the pivotal role of complement component C1q in this pathway detection and quantitation of both C1q protein and anti-C1q autoantibodies is described in Chapter 4.

Low serum complement component proteins C4 and C3 have long been identified as an important diagnostic laboratory test for SLE. Chapter 5 describes assays of these two analytes and investigates the relationship between complement component C4 serum concentrations and gene copy numbers at this intriguing complex genomic region and its relationship with known silencing CT insertion in exon 29 and the presence of the retroviral element HERV-K (C4) in intron 4 of the C4 genes. New insights are reported and under review by the Journal of Clinical Immunology.

Digestion of double stranded DNA from apoptotic cells is a critical step in apoptosis. It has been postulated that deficiency and/or variation at the DNASE I locus on chromosome 16, the major exogenous enzyme degrading double stranded DNA, may be important factors in the genetic predisposition to SLE. Chapter 6 reports studies of genetic variation at the DNASE I locus in Western a population of Australian SLE patients. These results have now been published also.

A final chapter is included comprising general discussion and conclusions, together with additional comments and possibilities for future research into this important, but still enigmatic, autoimmune disease.

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List of Abbreviations

2D4D	Second to fourth digit length
Ab	Antibody
α C1qab	Anti-C1q antibodies
AIRE	Autoimmune regulator
AS	Ankylosing spondylitis
B-cell	B lymphocyte cell
BSA	Bovine serum albumin
C1q, C1r, C1s	C1q complex
C2	Complement component C2
C2Q0	Complement component C2 null allele
C4	Complement component C4
C4A	Complement component C4A
C4B	Complement component C4B
C4Q0	Complement component C4 null allele
CD	Cluster differentiation
CD154/ CD40L	CD40 Ligand
CIDP	Chronic inflammatory demyelinating polyradiculoneuropathy
CNV	Copy number variations
CPG	Five prime-C-phosphate-G-three prime
CMV	Cytomegalovirus
ConA	Concanavalin A
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNASE	Deoxyrobonuclease
dsDNA	Double stranded DNA
dsDNA ab	Double stranded DNA antibodies
E2	17 β estradiol
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FB	Factor B
FcGR	Fc gamma binding receptor
FCS	Fetal calf serum
FE	Fetal estrogen
FT	Fetal testosterone
F:M	Female to male ratio
GCN	Gene copy number
GWAS	Genome wide association studies
HLA	Human leukocyte antigen
HW	Hardy Weinberg equilibrium
IFN	Interferon

IL	Interleukin
KS	Klinefelter's Syndrome
LD	Linkage disequilibrium
LN	Lupus nephritis
LPS	Lipopolysaccharide
mDC	Myeloid dendritic cell
mDC1	Myeloid dendritic cell 1
mDC2	Myeloid dendritic cell 2
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NOD	Non-obese diabetic
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCD	Programmed cell death
pDC	Plasmacytoid dendritic cell
RA	Rheumatoid Arthritis
RCCX	Complex comprising genes as follow on the MHC: Serine/Threonine nuclear protein kinase RP (STK 19), C4A &/or C4B, steroid 21-hydroxylase CYP21 (CYP-21) and extra cellular matrix protein tenascin X (TNX)
SET	Potein domain Su(var)3-9, Enhancer-of-zeste and Trithorax.
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T1DM	Type one diabetes mellitus
TCR	T cell receptor
TGF- β	Transforming growth factor-beta
Th	T helper
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor-alpha
Treg	Regulatory T cell
TREX1	3 prime exonuclease 1 (DNASE III)
ROS	Reactive oxygen species
UK	United Kingdom
VAST	Variant analysis of sequenced pedigrees
YAA	Y-linked autoimmune accelerating locus

List of Relevant Publications

- Establishment of gene copy number-specific normal ranges for serum C4 and its utility for interpretation in patients with chronically low serum C4 concentrations (Margery-Muir AA, Wetherall JD, Castley AS, Hew M, Whidborne RS, Mallon DF, Martinez P, Witt CS; 2014).
- Gender balance in patients with systemic lupus erythematosus (Margery-Muir AA, Bundell C, Nelson D, Groth DM, Wetherall JD; 2017).
- Anti-C1q antibodies concentrations by Elisa in systemic lupus erythematosus (Margery-Muir AA, Wetherall JD, Groth DM, Bundell C; 2017).
- Genetic variation at the DNASE I locus in an Australian cohort of SLE patients (Margery-Muir AA, Wetherall JD, Groth DM; 2017).
- Insights on the relationship between complement component C4 serum concentrations and C4 gene copy numbers in a Western Australian systemic lupus erythematosus cohort (Margery-Muir AA, Bundell C, Wetherall JD, Whidborne R, Martinez P, Groth DM; accepted for publication by Lupus (Sage) journal 2018).

Abstract

Systemic lupus erythematosus (SLE) is a polygenic, multifactorial autoimmune disease, characterised by autoantibody production, immune complexes and inflammation leading to multiple organ and tissue damage. It is postulated that aberrant apoptotic processing of cellular debris generates inflammatory mechanisms manifesting as signs and symptoms of SLE in genetically predisposed persons. This project focused on the interpretation of a panel of laboratory investigations used in the diagnosis and management of SLE in the light of this hypothesis. It is expected that the results obtained will assist clinicians in the diagnosis and ongoing treatment and monitoring of SLE patients in Western Australia.

In addressing this project, those characteristics of SLE that help define this unusual autoimmune disease were identified. In particular, it is suggested that laboratory tests used in the diagnosis and management of SLE should reflect mechanisms underlying immunopathology and genetic predisposition. Consequently, the review of the current literature focussed on the accepted female predominance of SLE, including a revision of its epidemiology, and the role of genetic deficiencies in the predisposition to SLE. This review included relevant research on twins, sex chromosomal abnormalities, genome wide association studies and possible environmental factors.

Human ethics approval, incorporating informed consent, was obtained for an investigation of 56 diagnosed and treated SLE patients and a cohort of 33 age/sex-matched healthy persons. A one-time collection of fresh peripheral blood permitted a flow-cytometry analysis of immune related cell phenotypes in all 89 persons tested. Intracellular cytokine synthesis of IL17 and the cytokine transforming growth factor beta (TGF β) was measured together with the serum levels of a panel of 13 inflammatory cytokines using a cytokine bead array assay. Contrary to expectations, Th17 cells were at very low frequencies in the SLE cohort. Synthesis of IL17 was increased in regulatory T cells and T cells expressing both the g and d markers ($\gamma\delta$ T

cells), the latter showing the highest relative serum proportions. SLE patients exhibited a reversal in the proportions of myeloid dendritic cell subsets 1 and 2 (mDC1 & mDC2) with increased mDC2 and correlated with the frequencies of the $\gamma\delta$ T cell subset. There was a relative increase in serum concentrations of the inflammatory cytokines IFN γ TNF α , MCP-1, IL-6, IL-8 and IL-18 in SLE patients relative to controls. It is clear therefore that the SLE patient cohort exhibited inflammatory signatures. We propose that the treatment regimens of the SLE patients in this study suppress TH17 cell numbers and another pathway involving the $\gamma\delta$ T cell/mDC2 axis maintains inflammation.

It is further hypothesised that defective apoptosis permits binding of complement component C1q to nuclear debris with generation of a neo-C1q antigen and subsequent initiation of an adaptive humoral response and anti-C1q antibodies (α C1q ab). Antibodies reactive with double stranded DNA antibodies (dsDNA) may also be produced by cognate mechanisms. Consequently, serum concentrations of C1q and complement components C3 and C4 were measured together with α C1q ab levels and correlated with dsDNA antibody concentrations from patient files.

It was observed that α C1q ab levels were elevated in SLE patients relative to controls, but all individuals tested manifested a positive result. α C1q ab levels correlated with dsDNA antibody levels in SLE patients, but not with serum C1q protein levels. It was concluded that measurement of α C1q ab was not specific for SLE but was useful for exclusion of SLE. It was noted that higher levels of α C1q ab were present in the serum of those SLE patients that exhibited co-morbidities and/or received multiple therapeutic treatments.

The generation of autoantibodies in patients with active SLE permits activation of the classical pathway of complement activation which is confirmed by the presence of low serum protein levels of complement components C2, C3 and C4. The results obtained in this study for C4 serum protein concentrations confirmed this expectation. However, deficiencies of complement proteins C1q, C2 and C4 are also well-known risk factors for SLE. To address this anomalous dilemma, quantitation of total gene copy numbers (GCNs) was performed for the multiple paralogous C4A and

C4B loci that may be present in an individual, together with the presence or absence of the HERV-K retroviral sequence that is present in many C4 genes. It was shown that for both the SLE and control cohorts serum C4 concentrations correlated with GCN. However, for SLE patients, variation in serum C4 concentrations precluded predicting serum C4 levels. This result confirms that consumption of C4 protein is responsible for low serum concentrations observed in persons with active SLE rather than deficient synthesis due to either lower C4A and/or C4B GCN. Furthermore, these results did not support low C4A GCN as a risk factor for SLE, although this view is often advocated.

Efficient apoptosis requires degradation of chromatin containing DNA by endonucleases. Defective DNA degradation due to endonuclease activity would be expected therefore to be a significant risk factor for SLE. Serum concentrations of the DNASE I enzyme were measured, together with typing of polymorphic variation at this locus on chromosome 16p13.3. DNASE I protein was present in all persons tested with no significant differences between concentrations in the SLE and control cohorts. Furthermore, of the many genotypes identified at this locus, there was no association between SLE and a specific genotype. These findings do not preclude DNASE I deficient individuals from manifesting SLE, but do not support observations showing some DNASE I genotypes are associated with SLE. We conclude therefore that genetic typing at the DNASE I locus is not useful in the diagnosis of SLE, or in predicting those individuals that are deficient in the synthesis of this important enzyme.

Finally, the experimental results obtained during this project have been published in a series of three papers. A shortened version of the thesis literature review has been published as a review of gender balance in patients with SLE. The thesis that follows includes copies of the experimental papers.

Chapter 1. Literature review: Gender balance in patients with Systemic Lupus Erythematosus

Part of the content and ideas in this chapter have been published in Autoimmunity Reviews (2017). Permissions for this article can be found in appendix B.1.1 and published article in B.2.1.

Margery-Muir A A. Bundell C. Nelson D. Groth, D M. and Wetherall JD. *Gender Balance in Patients with Systemic Lupus Erythematosus*. Autoimmunity reviews. 2017.

1.1 Abstract

Factors are reviewed that contribute to the contemporary view of a disproportionate prevalence and incidence SLE in females. Recent studies on the epidemiology of SLE report that global Incidences and prevalences of SLE for Caucasian and Black populations are of the order of 5.5 and 13.1 per year and 81 and 212 per 100,000 persons respectively. Both parameters displayed age dependent variation over a 90-year lifespan. The female to male (F:M) incidence of SLE varied with age, being approximately 1 during the first decade of life, followed by a sharp increase to 9 during the 4th decade, thence declining in subsequent decades before an increase during the 7th or 8th decades. A cognate review of SLE diagnosis in neonates revealed a F:M ratio of ≈ 1.2 , consistent with the epidemiology review and the sporadic nature of SLE. Notional estimates of disease duration showed a steady increase from a base level for both males and females. The linear trend line for males was always lower than the trend line for females, supporting clinical experience that SLE is a more severe disease in males. Over a 14-year interval ending in 2012, the notional duration of SLE increased from 10-15 years to 20-25 years, probably reflecting advances in diagnosis and clinical practice.

A metastudy of SLE concordance in twins revealed a 75% discordance in monozygotic twins compared to a 95% discordance in dizygotic twins confirming the importance of environmental factors in susceptibility to SLE. The elevated discordance in dizygotic SLE twins (and between siblings) suggests a role for the intrinsic genomic sexual dimorphism due to divergence of Y chromosome regulatory loci from their X chromosome homologues due to lack of recombination of mammalian sex chromosomes over evolutionary time.

Estimates were made of the incidences of SLE in males and females based on population data for nine autosomal deficiency loci of major effect, plus expected male prevalence associated with Klinefelter's syndrome and female prevalence associated with Triple X syndrome. These genetic abnormalities accounted for $\approx 4\%$ of female and $\approx 23\%$ of male Caucasoid prevalence and for SLE resulting in a F:M ratio of ≈ 0.17 . It may be deduced therefore that the impressive preponderance of SLE in

females arises from a combination of environmental triggers and susceptibility loci of relatively small effect acting between the interval from the mini-puberty of childhood to the peak of reproductive adulthood. It is in this cohort of females, and especially in the black population, that combinations of loci of minor effect acting together with environmental factors initiate defective apoptosis resulting in consequential autoimmune disease especially SLE. We postulate that because apoptosis is itself a very complex process, and defective apoptosis is an important contributor to SLE, there will be many combinations of susceptibility loci and environmental stimuli that can result in SLE (and other autoimmune disease(s)), of varying severity.

1.2 Introduction

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease characterised by the presence of autoantibodies against chromatin, including dsDNA, and inflammation of various organs, especially kidney and skin (recent reviews 13, 14). *Laboratory studies on cell-mediated inflammatory mechanisms were undertaken and are presented in Chapter 3, page 51.* For many years SLE has been considered an archetypal hypersensitivity state mediated by inflammatory processes following organ specific deposition of immune complexes with consequential activation of inflammation pathways including the complement system (14). The disease is managed by anti-inflammatory and immunosuppressive therapies that significantly reduce mortality rates, but do not effect cures (15).

The aetiology of SLE is unclear, however ineffectual clearance of cellular debris and chromatin during apoptosis has been proposed as the important effector mechanism in many instances and there is considerable evidence to support this hypothesis (reviewed by 16, 17 and 18). *Laboratory studies on anti-C1q antibodies relating to defective clearance of apoptotic cells and debris were undertaken and are addressed in Chapter 4, page 65.* The incidence of SLE is sporadic, however familial disposition is well described and is often associated with known deficiency states. The latter include deficiency states in the early acting components of the classical complement pathway (14) and TREX1 activity (19, 20).

The overall observed and widely reported gender imbalance in persons diagnosed with SLE is impressive. As estimated in this report, ~nine females are diagnosed with SLE for every male, although as will be seen this ratio varies between ethnic populations and with age of the test population. It is proposed the extraordinary preponderance of SLE in females of reproductive age provides important clues to the underlying triggers that lead to SLE in susceptible persons. This review will seek to identify those factors that contribute to the disproportionate prevalence SLE in females.

1.3 Incidence and Prevalence of SLE in Human Populations

There have been many reports of the incidence and prevalence of SLE in diverse human populations. These studies have shown higher prevalences in women of reproductive age (20-40 years) and in women of black or Asian ancestry (reviewed by 21, 22). Virtually all studies report an impressive female to male (F:M) ratio. Estimates used in this review of incidence and prevalence of SLE for Caucasoid and Black populations are based on the studies of (23, 24) because they discriminate clearly between these two ethnic populations. Mean incidences and prevalences of SLE for Caucasian and Black populations are 5.5 and 13.1, and 81 and 212 per 100,000 persons respectively. The F:M ratio is 9 for both ethnic groups. Data are also included of estimates from two very recent European studies of ethnically mixed populations from the United Kingdom (UK) (25) and France (26). A fifth report (27) includes data for a small population of indigenous Australians. A summary of the prevalence and, incidence rates and F:M ratios from these studies is shown in Table 1.1. It is in the nature of epidemiological research that results are sensitive to the methods and definitions used. Differences between the four reports summarised in Table 1.1. reflect variations in database composition and disease definitions as well as the composition of the populations interrogated together with any environmental factors that may be involved. Despite these limitations a consistent epidemiology of SLE emerges.

Both incidences and prevalences are higher for females than for males, and the increased susceptibility of individuals from African descent for SLE is clearly apparent.

It is interesting to note that the higher incidence and prevalence rates of SLE reported by (27) for indigenous Australians in North Queensland are similar to those for European and North American Individuals from African descent. Given the long isolation of indigenous Australians, this observation very likely reflects their more closely shared African genetic ancestry.

The gender imbalance ratio based on prevalences is ≈ 10 for all studies. The data of Rees and Arnaud (25, 26) included variation in both incidence and prevalence rates as a function of age by decade and it seems clear that SLE may be newly diagnosed in all decades of life. The pronounced increase in incidence of SLE in females between 20 and menopause is clearly seen and contrasts with the slower but steady increase in male diagnosed SLE over their lifetimes. These observations translate into highly variable F:M ratios based on incidences (and prevalences) over a life time and probably account for the variation in ratios often reported in the literature. There are two peaks in the F:M ratio based on incidences – these occur in the third decade (20-29 years) due the increased susceptibility of females of reproductive age and again in persons older than 79 years. A simplistic measure of disease duration (in years) is the ratio of prevalence to incidence. We have used the data of Rees et al (25, 28) to show this graphically in Figure 1.1 for both males and females.

Figure 1.1 shows that over a human lifetime disease duration is shorter in males than females which is consistent with the many reports that conclude SLE is more severe in males and especially in children (29-31). The steady increase in disease duration over adulthood likely reflects less severe disease profiles and rapid improvements in the diagnosis and treatment of this disease with consequent increased longevity. This point is reinforced by Rees et al (25) who estimated temporal incidences and prevalences for their mixed UK population over the interval 1999 to 2012. Disease duration estimates for these data are presented in Figure 1.2 and show a steady increase in disease longevity.

Jianguang Ji and colleagues (32) reviewed the gender specific incidence of autoimmune disease in 403757 subjects registered in the Swedish national disease databases. Their conclusions are generally supportive of the view reported herein

that female predominance of autoimmune diseases may be less striking than previously believed. They also reported that the median age of onset of SLE was 49 years and 58 years for females and males respectively; these values are in close agreement with those of Rees et al., (25) whose data for the year 2012 implies values of 52 and 62 for females and males respectively in the UK populations. In the latter case the median age of onset of SLE was greater for females than males only for the interval from the 3rd to 6th decades of life.

1.4 Imbalance of sex ratio in offspring of SLE mothers

A possible explanation for the female preponderance of SLE is loss of male fetuses during the pregnancies of mothers with SLE. This hypothesis was initially addressed by Oleinick (33) who reported a significant decrease in the ratio of live born male siblings to total live born siblings to SLE diagnosed parents. In 1998 Wallace (34) speculated the reason for the rarity of male lupus could be presence on the Y chromosome of a "lethal gene" leading to miscarriage of a male fetus, who, if born, would be at a very high risk of developing SLE. Moorthy and colleagues (35) studied a related question by asking whether children with SLE have fewer male siblings than would be expected. They reported the proportion of male siblings for SLE probands is 0.39, consistent with the families of children with SLE being deficient of male children. The same phenomenon was not observed in the children of probands with pauci-articular onset juvenile rheumatoid arthritis (PaJRA) which also exhibits female preponderance and systemic juvenile onset rheumatoid arthritis (SoJRA) which does not exhibit a female preponderance (35).

Aggarwal *et al.* (36) determined the sex distribution of 6056 siblings from 2579 women diagnosed with SLE on samples from patients registered with the "The Lupus Family Registry and Depository" (37). A significant excess of female children relative to male children was observed in this comprehensive study; we estimated the decreased proportion of male siblings in families with an SLE mother to be approximately 13%. A more recent large study has not confirmed the reports described above. Dar and colleagues (38) used the records of the Maccabi Health Services (Israel) to determine the proportion of live born males in 380,472 offspring

of women free of rheumatoid arthritis (RA), SLE and psoriatic Arthritis (39) and in 182,073 women with at least one of these three diseases. Patients with SLE or RA did not differ from the general population by the sex of their offspring. It is difficult to reconcile the differing conclusions from these two important studies. It is possible that the selection of SLE patients in the Israeli Maccabi Health Services database was too sensitive and the inclusion of dubious cases may have obscured a deficiency of male offspring.

In a related study of male only SLE, Aggarwal and colleagues (40) described five families with only male SLE. They observed that white men with SLE were five times more likely to have a child with SLE than were white women with SLE. This likelihood was not observed in Individuals from African descent. Fluorescent *in situ* hybridisation studies in the five families with only male SLE failed to detect an equivalent of the “Y-linked autoimmune accelerating locus” designated *Yaa*. The *Yaa* locus was initially described by Murphy and Roths as a Y chromosome locus that accelerated SLE disease development in male BXSB hybrid mice (41). It is now known that *Yaa* arises from a translocation of X-linked genes onto the Y chromosome of the BXSB mice leading to a duplication of *Ms131*, *TLR7*, *TLR8*, *Tmsb-4x* and *Rab9* loci (42, 43). As described above, the elevated F:M ratio for SLE only occurs during the reproductive stages of a woman’s life and the gender ratio of neonates and children diagnosed with SLE is much closer to unity. These observations are inconsistent with preferential male fetal loss in SLE.

1.5 Genetic factors affecting the gender ratio of SLE in humans

1.5.1 Twin studies

Many reports have demonstrated a familial nature of SLE. However twin studies have shown only 25-30% concordance between identical twins and little or no concordance between fraternal twins. Interestingly, our review of 18 case reports and studies of twins with SLE between 1975 and 2013 revealed a remarkable discordance rate. Indeed, these reports indicate total discordance rates in

monozygotic and dizygotic twins of 69% and 96% respectively. In calculating the overall rates for monozygotic and dizygotic twin sets, we find that 75% are discordant for SLE and 25% are concordant only. The summary of individual data and references for each study are shown in Table 1.2. Discordance between monozygotic twins is usually attributed to variation in environmental factors either in utero or within the interval between neonatal life and age of diagnosis of SLE. Incomplete information and variation between studies prevented reliable estimates of the F:M ratio of the twin pairs reported in Table 1.2.

Only a few of the twin studies in this table reported similarity of symptoms, outcomes and times of diagnosis/onset (44-46). In most instances, there was considerable variation in pathology, serology, and time of onset (47-49). Indeed, Kuroda and colleagues (47) reported a set of twins who still lived together at time of onset of disease; despite MHC class I and II similarity, the diagnosis of SLE for each twin differed by three years with only one twin manifesting a lupus psychosis. In a discordant case, both twin sisters presented with *Myasthenia gravis* and both received a thymectomy 14 years apart; the twins were diagnosed two years apart and followed for 28 years. The concordant twins reviewed by Del Boz and colleagues (45) were males with Klinefelter's XXY karyotype, similar pathologies and age of diagnosis.

Fraga and colleagues (50) studied epigenetic profiles in monozygotic twins and observed distinct epigenetic patterns in each twin. They showed these differences were associated with age, the time spent together and their medical history. Moreover, gene expression profiles showed a four-fold differential expression in older twins and a higher number of overexpressed genes and hypo-methylation that may lead to increased transcription and activation of these genes. It seems clear that although twins are viewed as genetically identical, distinct epigenetic signatures reflecting environmental differences may generate distinct phenotypes that could account for discordance in autoimmune diseases, including SLE.

1.5.2 Neonatal SLE

Nineteen reports of neonatal SLE between 2013 to 2016 were reviewed and are summarised in Table 1.3. In 114 neonates with SLE there were 63 females and 51 males to give a F:M ratio of 1.2. This result is consistent with the data presented in Figure 1.1 showing the incidence of SLE in male and female children in the first decade of life. Of the 114 neonates with SLE the mothers of 46 were not recorded as having tests or symptoms of autoimmune disease, especially SLE. This result is consistent with sporadic SLE being frequent.

1.6 SLE associated with chromosomal mutations

The increased incidence of SLE in females relative to males suggests an important role for the X chromosome in the predisposition to SLE. Nondisjunction of the X chromosome during meiosis gives rise to males with an XXY karyotype and subsequent abnormalities referred to as Klinefelter's syndrome (KS). Although reported cases are low (about 30 cases between 1970 and 2009), the prevalence of SLE in males with KS is increased ≈ 14 times relative to normal males (11). The disease phenotype in KS males is similar to that in females. In a follow up study to that of Scofield *et al.* (11), Dillon and colleagues (51) reported that 7 of 286 men with SLE from the Lupus Family Registry and Depository (37) manifested abnormal karyotypes that included 47XXY. In contrast to non-KS men with SLE the disease symptoms of the KS men with SLE were less severe and did not include nephritis. Further, it is clear that not all KS males develop SLE. Men with KS who develop SLE behave as a cohort more like women with mild SLE. The prevalence of KS in Australia is approximately 1115 per 500,000 males (52) with most KS patients being initially identified as adults presenting with infertility.

Harris and colleagues extended these findings by reporting that the KS karyotype was more frequent in males diagnosed with Sjogren's syndrome than in either control populations or rheumatoid arthritis patients (53); they also confirmed excess KS in men diagnosed with SLE. In a cognate study Liu *et al.*, (54) reported an increased prevalence of SLE and Sjogren's syndrome in a cohort of females with another

chromosomal mutation – the 47XXX karyotype, providing further evidence for dosage effects reflecting X chromosome copy number. The prevalence of 47XXX in patients (females) with SLE was estimated to be 0.25%. The prevalence of SLE in females with Turner’s syndrome (characterised by the 45XO karyotype) seems to be diminished relative to normal females. Only three cases of SLE in women with the 45XO karyotype have been reported possibly because karyotyping is not usually performed in these women (12, 55, 56), although the Ruas study also described neonatal SLE in a 45XO female infant.

1.7 Genomic sexual dimorphism - influence of the Y chromosome

Recent sequencing and phylogenetic analysis of the human Y chromosome has provided important new information on intrinsic differences between male and female cells (57, 58). It is now understood that the Y chromosome includes a complement of regulatory genes (UTX/UTY, EIF1AX/EIF1AY, ZFX/ZFY, RPS4X/RPS4Y1, KDM5C/KDM5D, DDX3X/DDX3Y, USP9X/USP9Y and TBL1X/TBL1Y) that have diverged from their X chromosome homologues due to lack of recombination of mammalian sex chromosomes over millions of years. The homologues of these regulatory genes on the X chromosome also remain functional on the inactivated X chromosome in females so that they are present in both sexes in two copies, albeit that the Y chromosome variants have diverged significantly from their X chromosome counterparts. Notwithstanding the powerful differential influence of sex hormones on male and female tissues, differences between the divergent regulatory homologues shared by the sex chromosomes create a sexual dimorphism manifested as X and Y-encoded protein isoforms in diverse human tissues. David Page (see 57) has speculated “whether this dimorphism has a role in diseases, outside the reproductive tract, that occur with greater frequency or severity in males or females”. These studies do not explain however the frequent discordance of monozygotic twins with SLE, including those also manifesting Klinefelter’s syndrome (see discussion above). Genomic sexual dimorphism may be further complicated by non-random inactivation of the X chromosome in a female cell – a phenomenon called skewed X

chromosome inactivation. Kast was the first to postulate a role for X-inactivation chimerism in female-prevalent autoimmune disease (59). Jeffrey Stewart extended this hypothesis to propose that the discordance rate of SLE between monozygotic twins may be explained by such twins manifesting different X-inactivation patterns, possibly in a tissue specific manner (60). There seems to be no published evidence to support a role for skewed X inactivation in SLE.

1.8 Mitochondrial DNA and SLE

The 16 Kb circular mitochondrial genome encodes thirteen proteins of the inner mitochondrial membrane respiratory complex; it is present in multiple copies and inherited matrilineally (61). The major function of mitochondria is the production of energy by ATP reduction that is essential for all intracellular processes (61, 62, 63, 64, 65). ATP from mitochondria is also important in promoting immune responses via inflammasome activation and in regulating apoptosis (66). Apoptosis is a programmed form of cell death involving the degradation of cellular constituents by a group of cysteine proteases called caspases. The caspases may be activated through the mitochondrial mediated intrinsic pathway (67) or via the “death receptor” mediated extrinsic pathway (68). The intrinsic pathway of apoptosis is initiated by intracellular signals resulting in permeabilisation of mitochondria with release of cytochrome c into the cytoplasm and subsequent apoptosome activation of the caspase cascade (67, 69). Perl (70) has recently reviewed evidence confirming increased production of reactive oxygen species (ROS) and consequent oxidative stress in peripheral blood lymphocytes (PBL) from patients with SLE; mitochondria in T cells from patients with SLE exhibit dysfunction characterized by elevated mitochondrial transmembrane potentials (71, 72). These events may lead to aberrant apoptosis, oxidative stress, and inflammatory responses (73, 74, 75). Hence it is possible that variation in maternally transmitted mitochondrial DNA may predispose to SLE and other inflammatory autoimmune disorders. If genetic variation in mitochondrial DNA is a candidate locus for predisposition to SLE It is unknown whether there are gender specific differences.

1.9 Inherited autosomal deficiency states predisposing to of SLE

There have been many reports of SLE in kindred groups manifesting an autosomal deficiency state. In these families, the susceptibility to SLE usually reflects a strong association with a specific genetic locus (Table 1.4). The best described and longest known autosomal loci of major effect in predisposition to SLE encode complement proteins C1q, C1r, C1s, C2, C4A and C4B. Of these, genes encoding C2 and both C4A and C4B (as well as Factor B) occur within the central region of the human MHC on chromosome 6p21.3 (reviewed by 14). It is notable that while these associations between the complement component (complete) deficiency state and SLE are generally strong they are not absolute. The percentage of persons with a complete deficiency state that are concordant for SLE varies from a low of 57% for C2 through to over 90% for C1q (Table 1.4).

C1q and C1r deficiencies are very rare and associated with early childhood onset of SLE. Stegert (76) and Van Schaarenburg (77) reported the median age of SLE onset was five and nine years old respectively. Van Schaarenburg (77) showed early age of death in their C1q deficient SLE patients (F:M ratio of $\approx 1.3:1$) with a mortality of 20% at time of follow up. The C2Q0 allele has been estimated to have a frequency of 0.02 in Caucasian populations resulting in 0.04% of persons with homozygous C2 deficiency states (78-80). Of these only 10% manifest SLE with a sibling concordance rate of approximately 50% and an F:M ratio of ≈ 7 , the latter figure being closer to the overall ratio for all SLE patients (see Table 1.1).

Humans have two forms of the complement C4 gene designated C4A and C4B. Both loci encode isotypic forms of C4 protein (C4A and C4B) each with distinct biochemical properties and each displaying multiple electrophoretic variants reflecting allelic polymorphisms (81, 82). Null alleles designated C4AQ0 and C4BQ0 exist at both loci as a result of several well-defined mutations (83-85). A further level of complexity arises from the propensity of this chromosomal segment to duplicate resulting in as many as four distinct replicons per chromosome each containing either a C4A locus or a C4B locus (81, 82). Males and females share this complexity and the distributions

of serum levels for both C4 proteins is similar in males and females (86). Wisnieski and colleagues described a rare three-generation kindred with incomplete C4 deficiency resulting from hyposynthesis of C4 protein controlled by an unidentified autosomal dominant non-MHC linked locus (87). Null alleles of C4 genes were not involved and hyper-catabolism of C4 protein was excluded. In this study of 28 persons with 10 persons exhibiting low C4 serum concentrations only the proband was diagnosed with SLE. This report supports the argument that low C4 serum levels per se are neither sufficient nor necessary for the development of SLE. Reliable population data for the frequencies of C4AQ0 and C4BQ0 null alleles is limited, although in Caucasians the frequencies of the C4AQ0 and C4BQ0 alleles are reported as 1% and 3% respectively (14) complete C4 protein deficiency is therefore very rare. If these loci manifest Hardy Weinberg equilibrium the expected frequency of C4A and C4B protein deficient persons would be expected to be less than 1 in 1 million. Both *C4A* and *C4B* loci, together with the complement Factor B and complement C2 loci are located within the central region of the MHC and behave as a genomic block within which recombination is restricted. Hence it is not meaningful to consider these two loci individually when assessing their contributions to male and female differences in susceptibility to SLE. *Laboratory studies on complement C4 loci were undertaken and are presented in Chapter 5, page 77.*

Other loci predisposing to SLE are the inhibitory Fc gamma binding receptor (FcGR3B), endogenous DNASE (Trex1) and exogenous DNASEs I and II. Fc receptors link humoral and cellular immunity by facilitating phagocytosis and antibody dependent cell mediated cytotoxicity and are important in modulating the immune response (88). The human *FcGR* locus on chromosome 1q23 is subject to copy number variation (CNV) (4, 89, 90). This genomic region contains the *FcGR3B* locus and paralogous genes *FcGR2A*, *FcGR3A*, *FcGR2C*, and *FcGR2B*, encoding low-affinity Fc gamma receptors (4). The inhibitory receptor gene, *FcGR2B*, exhibits a polymorphism within the trans-membrane domain that is associated with SLE (5). Several groups have reported that diminished efficiency of the FcGR complex by either mutation or deletion is associated with SLE (4, 89, 90). Mutations in the Fc region of immunoglobulin heavy chains that bind complement C1q may also reduce

the efficiency of removal of complement activated antibody-antigen complexes thereby precluding complement activation and phagocytic removal of the complexes.

Patients with SLE demonstrate defective clearance of cell debris, which could trigger autoimmune responses (91). Hence mutations in genes involved in the digestion of dsDNA and chromatin have been implicated in the development of SLE (1, 3, 6, 20, 92-100), especially in patients with early age of onset and lupus nephritis (3). TREX1 (DNASE III) is the major 3'-5' DNA exonuclease within mammalian cells. *TREX1* is located on chromosome 3p21.31 and consists of a single exon encoding a homodimeric 314 amino acid polypeptide chain (7). TREX1 is an important component of the SET protein, a multitasking protein complex that associates with the endoplasmic reticulum and translocates to the nucleus in response to oxidative stress. In association with SET, TREX1 causes single-stranded DNA damage during caspase-independent apoptosis activated by granzyme A (7). Crow and colleagues have described an IFN-driven inflammatory disease associated with TREX1 loss in human patients (Aicardi-Goutieres syndrome) that is characterised in part by an autoimmune syndrome similar to SLE (7, 101).

Lee-Kirsch *et al.*, (6) reported that nine of 417 SLE patients from the UK, Finland and Germany (males and females not specified) manifested *TREX1* mutations with no mutations in their control cohort. More recently Ellyard and colleagues described a four-year-old female with cerebral SLE caused by a rare homozygous mutation in *TREX1* (102). Namjou and colleagues reported that the estimated frequency of *TREX1* mutations in their mixed ethnicity SLE cohort was 0.5% (99). These findings are consistent with mutant forms of TREX1 having impaired exonuclease activity and therefore predisposing to inflammatory diseases by reduced degradation of dsDNA during apoptosis. Another recent study (9) provides important insights into the digestion of DNA containing microparticles (formed from reverse transcribed retro-elements) and its role in preventing autoimmune disease. DNA from apoptotic cells is degraded by the intracellular enzyme DNASE2, whose deletion in mice causes IFN-driven auto-inflammation (103). These rare but highly penetrant causative mutations are not detected in genome wide studies which may explain part of the missing

heritability of SLE as well as provide insights into disease pathogenesis. We are unaware of any gender bias in the expression of *DNASEs*.

It is also of considerable interest that endogenous DNASE activity is present in blood from healthy persons. DNASE activity is increased in the serum of SLE patients experiencing a relapse of disease, but not in healthy controls (104). Barcelos-Barra and colleagues showed that DNASE activity in blood plasma is inhibited by the anti-coagulant EDTA which more closely mimics the physiological state of circulating blood (105). The genetic basis of DNASE activity in human blood has not been described. *Laboratory studies on DNASE I locus polymorphisms were undertaken and are presented in Chapter 6, page 90.*

Crow et al., (106) has suggested that SLE is a heterogeneous collection of individually rare genetically distinct disorders sharing a common inflammatory pathology. These deficiency states, although relatively rare, go some distance in accounting for the genetic complexity of SLE predisposition (see also Table 1.4). Further, these autosomal deficiency states are expressed in both sexes and, as expected, do not contribute to the high female prevalence in SLE patients.

1.10 Genome wide association studies of SLE

Genome wide association studies (GWAS) interrogate individual genomes of persons with a specified disease or trait for the presence or absence of SNPs exhibiting significant linkage disequilibrium. GWAS' are complex, require large numbers of test individuals and well-defined SNP panels to achieve useful outcomes and are more difficult to analyse when SNPs on the single copy sex chromosomes are included (107). The latter difficulty is especially relevant for SLE where the incidence shows a strong female bias.

For example, Graham and colleagues (108) have performed a review of the literature and meta-analysis of published candidate variants from 1310 SLE cases of North Americans of European descent and 7589 controls. Many of the candidate loci identified also functioned in B-cell signalling and development, through toll-like receptors seven and nine, as well as neutrophil function which are believed to be

important in the development of SLE. Candidate loci identified by GWAS occur on both autosomes and the X sex chromosome in humans. Seventeen variants were observed that accounted for about $\approx 30\%$ of the genetic predisposition to SLE (108). Candidate loci on autosomes should contribute to SLE predisposition in both males and females unless there is a sex specific regulatory mechanism in place.

Two more recent GWAS in SLE cohorts have been published and these both confirm and extend many earlier studies (109, 110). Bentham *et al.*, (109) undertook a comprehensive GWAS on 7219 SLE cases and 15991 controls of European ancestry and mapped 43 susceptibility loci that included 10 new associations. The susceptibility loci included an over-representation of 16 transcription factors. Sun and colleagues (110) reviewed a GWAS' on 4478 SLE cases and 12,656 controls of East Asian ancestry in which the incidence of SLE is greater than Caucasians. Ten new loci and 20 previously known SLE susceptibility loci were identified with many of these involved in immune cell (T and B) functions. Sun *et al.*, (110) also estimated the total heritability of SLE in Asians accounted for by their study to be $\approx 24\%$. So *et al.*, (111) used a statistical method to estimate the heritability of 10 complex diseases (including SLE) from GWAS and concluded that GWAS underestimated heritability; their estimate for SLE was 13% heritability. It is of interest that neither study described above identified TREX1 as a susceptibility locus for SLE, although as reported by Ellyard *et al.*, (3) this locus has a rare allele that when homozygous causes SLE. In our view, it is likely that a greater proportion of the heritability of SLE as well as other autoimmune disorders will be attributable to rare alleles of major effect, including also the role of compound heterozygosity where two distinct mutant alleles create a disease predisposition reflecting a functional deficiency state.

GWAS are based on linkage disequilibrium estimates between susceptibility loci and a specified trait, whether that trait is a disease diagnosis or a productivity measure in agriculture. The utility of this method is diminished by the presence in human genomes of extended haplotypes which while polymorphic themselves remain stable due to suppressed recombination (112). Given the inherent low efficiency of GWAS' and this latter complication, the future of GWAS in the study of complex diseases is problematic. The development of affordable powerful highly parallel genome

sequencing methods now permits a more direct approach to identification of susceptibility loci for complex diseases in general. A subset of this technology, abbreviated to VAST (variant analysis of sequenced pedigrees), has been described by Field *et al.*, (113) and holds much promise for identifying causal loci that may explain some of the discordance shown by identical twins with SLE.

1.11 Estimated prevalence of SLE in males and females due to autosomal loci of major effect and Klinefelter's syndrome

The prevalence of SLE in males and females due to chromosomal mutations of the sex chromosomes and known autosomal loci of major effect is estimated as shown in Table 1.4. This table records prevalences of SLE in a Caucasoid population of one million persons (males = females). There is a paucity of data for the prevalences of most of the autosomal deficiency states that are strongly associated with SLE, although it is clear these are mostly rare deficiency states. To overcome this problem, we have therefore assumed population prevalences for complete C1q, C1r, C1s, C4A+C4B, and TREX1 (DNASE III) deficiency states as one in one million, based on inferences from the literature and consider these conservative. Other prevalences are derived from the references shown in Table 1.4.

As shown in Table 1.4, these genetic predispositions of major effect account for a greater proportion of male SLE diagnoses than female. Further, based on the differing age dependent incidence and prevalence profiles for SLE most of these male diagnoses occur within the first two decades of life, or later in life. The impressive female preponderance of SLE prevalence arises therefore from the dramatic increase in females diagnosed with SLE in the 3rd and 4th decades coinciding with the reproductive years. If preconditioning *in utero*, or neonatal hormonal status, increase susceptibility to SLE in these cohorts, it is in these females that the underlying mechanisms and environmental triggers should be sought. Several possibilities have been recognized.

It is interesting to compare the main features of SLE with those of ankylosing spondylitis (AS), another autoimmune disorder of unknown aetiology in which the F:M ratio strongly favours males. It is an autoimmune seronegative spondyloarthropathy that exhibits a strong association the class I HLA-B27 allele (114, 115). HLA-B27 occurs in more than 80% of the European Caucasians diagnosed with ankylosing spondylitis relative to eight percent in the normal population reviewed by (116) to give an odds ratio of ten. Concordance of AS in identical twins of more than 90% has been reported reviewed by (116). The F:M ratio for incidence of AS is approximately one in the 18-29 year old individuals, and doubles by the 30 to 39 year old (1.97) and steadily decreases to 1.38 in the 80 plus year cohort (117). However, the prevalence F:M ratio of AS is approximately less than 0.025% in the 16 to 19-year-old group and steadily increases to 0.35% in the 55 to 59-year-old and above (117). Only four cases have been reported of AS in males with KS (118-121). Armstrong *et al.* noted that their AS patient with KS seemed to have a more female-like disease phenotype which is similar to the female disease phenotype observed in KS males with SLE. Interestingly, there are only four reports also of AS occurrence in females with Turner's syndrome (122-125) of which the report by Sandhya and colleagues (124) is describing a case of juvenile AS. GWAS on AS individuals have found about 30 genetic associations with autosomal loci but do not seem to have found any association on the X and Y chromosomes IGAS 2013; (126).

1.11.1 Gender differences in early microbiome development and immune responsiveness and predisposition to SLE

Sex based differences affecting both the innate and adaptive immune responses in humans are well known (reviewed by 127). It is believed that these contribute to differences in the pathogenesis of infectious diseases and responses to viral vaccines in males and females, and the increased susceptibility in females of autoimmune diseases especially during their reproductive years (127, 128). Of interest in this review is the study of Gaudreau and colleagues (129) who reported increased mucosal immune responsiveness of female (SWR x NZB) F1 (SNF1) mice compared with their male counterparts; female SNF1 mice are especially susceptible to SLE like

symptoms including proteinuria (130). Female SNF1 mice not only showed profoundly higher CD154, immune cell densities, but also carried large numbers of interleukin (IL)-17-, IL-22- and IL-9-producing cells in the *lamina propria* compared to their male counterparts. The intestinal mucosa of female SNF1 mice expressed higher levels of a large array of pro-inflammatory molecules, including type 1 interferons and TLR seven and eight even before puberty. This potential link between the immune response of the gut mucosa and SLE suggests that gender specific responses to environmental or dietary antigens may contribute to the gender imbalance of SLE incidence in humans. A further intriguing link between the mucosal immunity and SLE comes from the report by Markle and colleagues that early-life microbial exposures influence sex hormone levels and modify progression to autoimmunity in the non-obese diabetic (NOD) mouse model of type 1 diabetes in an androgen receptor dependent mechanism (131). The role of microbiota colonization in early life in modulating immune responsiveness has been reviewed by Gensollen et al. (132).

There is a clear role for hormonal factors and environmental interactions in the manifestation of this sexual dimorphism but possible molecular mechanisms are poorly understood. Two recent reports on regulation of immune responses by sex steroids have shown independently that expression of the autoimmune regulator locus (*AIRE*) is decreased in the female thymus relative to the male thymus especially during pre-puberty and the interval known as the mini-puberty of infancy (133, 134). Dragin and colleagues associated diminished *AIRE* expression with estrogen-induced gene methylation, while Zhu and colleagues followed the finding of increased male *AIRE* expression by demonstrating that the androgen receptor binds the *AIRE* promoter and increases *AIRE* expression (both mRNA and protein). In both studies, *AIRE* expression affected thymic expression of tissue-specific antigens. Finally, both groups showed that susceptibility to autoimmunity disease (in murine models of multiple sclerosis and thyroiditis) depended on sex steroid production in an *AIRE*-dependent manner. These ground-breaking studies provide renewed interest in the role of *in utero* sex steroid exposure as a predisposing factor to SLE.

The view that sex hormones contribute indirectly to SLE predisposition is supported by rapid increase described previously in the incidence (and prevalence) of SLE in post-pubertal women relative to both pre-pubertal males and females. It is known that androgen levels in female SLE patients are lower than in control groups regardless of treatment regimen (135).

1.11.2 Evidence for an elevated in utero androgen receptor environment in SLE predisposition

In humans, the ratio of 2nd to 4th digit length (2D4D) is sexually dimorphic with the mean 2D4D being longer in females than males. Over a decade ago Lutchmaya and colleagues (136) showed that the 2D4D ratio on the right hand exhibited a significant negative association between fetal testosterone to fetal estrogen concentration ratios (FT/FE) in amniotic fluid that was independent of sex. More recently Manning and colleagues have shown that this trait reflects the sex hormonal environment in utero, specifically the relative proportions of androgen versus estrogen receptors on the tissues of the 2nd and 4th digits respectively and is believed to be fixed early in development (137, 138). Doe and colleagues (139) measured 2D4D ratios in 100 patients with SLE each fulfilling the American College of Rheumatology criteria and 200 controls without SLE (each cohort contained equal numbers of males and females). Their study showed that patients with SLE had decreased 2D4D ratios relative to controls thereby providing indirect evidence that the SLE patients experienced high prenatal testosterone and low prenatal estrogen. Interestingly, men with KS, in which the prevalence of KS is similar to that observed in women, had 2D4D ratios similar to normal women and is consistent with the observations reported above that SLE in KS males is often less severe than in non-KS males and more closely resembles SLE in women of reproductive age (140). A study of men with and without multiple sclerosis also showed a decreased 2D4D ratio in men with the disease and concluded that low androgen exposure during the pregnancy could be a risk factor for multiple sclerosis (141). There is a large number of sexually dimorphic traits that show an association with the 2D4D ratio (see for example <http://www.handresearch.com/news/digit-ratio-finger-length.htm>). It seems that

the lower 2D4D ratio seen in female SLE patients offers support for elevated androgens during pregnancy being a risk factor for SLE that may act synergistically with physiological synthesis of sex hormones during prepubescent development, especially in females.

1.12 Sex hormone effects on disease

Human immune responsiveness sex-based differences affecting both the innate and adaptive immune responses are well described. It is believed that these contribute to differences in the pathogenesis of infectious diseases and responses to viral vaccines in males and females, and the increased female susceptibility to autoimmune diseases, especially during their reproductive years reviewed by (127). The rapid increase in the incidence (and prevalence) of SLE in post-pubertal women relative to both pre-pubertal males and females described above reinforces this view.

Hormone studies in SLE have shown increased up-regulation of immune plasmacytoid dendritic cells (pDCs) and co-stimulatory molecules by a collaboration of 17β estradiol (E2) and toll-like receptor 9 (TLR9) CpG ligand (142-145). In addition, immune cells express the estrogen receptors (ER) alpha and beta (α & β) that effect cell responses to estrogens such as 17β estradiol (146-148). Therefore, it has been postulated that E2 in the presence of the CpG ligand might stimulate pDCs to produce large amounts of IFN α and increase B cells viability as well as their release of auto-antigens (142). Other groups have demonstrated collaboration of E2 and IFN α which lead to prolonged survival of auto-antigens (149) or by an IFN α independent mechanism (150). On the contrary to the human studies, experiments in lupus mice models have not demonstrated specific effects from hormonal secretions (151). These reports suggest that hormonal regulation by itself is not pathognomonic of SLE. Other factors, for example dis-regulation of X-linked genes may influence disease outcomes. CD40 ligand (CD154) and TLR7 (HGNC:11935; HGNC:15631 respectively, NCBI) are two genes involved in cellular pathways as described above.

1.13 Other environmental factors

Despite well delineated genetic risks factors, serological, cellular, MHC phenotype, hormonal functions as seen previously in this review, it is clear that other factors, clearly environmental, must play a critical role in the outcomes of SLE in individuals.

There is no well-defined association between SLE and specific infectious agents, with the possible exception of the Epstein Barr virus (152). Several studies have demonstrated the presence of EBV antibodies in patients with SLE (153-155). Since EBV is a dsDNA virus it is clearly possible that impaired degradation of intracellular viral DNA could result in impaired apoptosis of infected cells thereby mimicking an important predisposing factor for SLE and other inflammatory disorders.

Deficiency of vitamin D correlates inversely with disease activity index in patients with lupus (156-159). Other environmental factors, may also play a critical role in the predisposition and outcomes of individuals with SLE. These include climate/temperatures and altitude (160-162), seasonal changes and UV exposures (162-165), paints and colour dyes, film development, polishes (nail) (161), insecticides (166), exposure to compounds such as Polychlorinated biphenyls/dibenzofurans (167). Increased risk of SLE associated with exposure to silica from dust and industrial urbanisation has also been reported (161, 168-172). Exposure to this compound increases the risk to SLE and rheumatoid arthritis up to eight-fold (172), and longer exposure increases the risk (171).

Hence, the different environmental factors might trigger SLE pathology in susceptible individuals who possess other risk loci as mentioned so far herein.

1.14 Conclusions and discussion

As described herein, recent epidemiological data shows that SLE may be first diagnosed in neonates and at all ages until old age. In contrast to males, the incidence and prevalence of SLE increases dramatically in post pubertal females and remains high during the reproductive years reaching peaks between the third and fifth decades. The F:M ratio for incidence, and hence also prevalence of SLE, varies

markedly with chronological age and ranges from ≈ 1 to 10 in figures used in this paper. The often quoted value of a F:M ratio of ten applies only over the third to fifth decades of life. This report shows that for children under ten years the F:M ratio is ≈ 1.2 and for elderly adults gradually decreases to ≈ 3 . The latter figure is probably influenced by the increased longevity of females relative to males and the more severe disease often seen in males. It is noteworthy that age dependent prevalence of SLE divided by the age dependent incidence of the disease shows a steady increase over ten decades for both females and males. This ratio is a notional measure of the average duration of disease and is always higher in females than males. This observation supports clinical reports that SLE is a more severe disease in males than females with shorter survival times.

The markedly increased F:M ratio during the third to fifth decades in women strongly supports a sex hormone mediated predisposition to the incidence of SLE. A similar observation in males with KS, together with a more female like profile of SLE reported in KS males, strengthens this conclusion. An explanation for this observation is likely to be found in the sex hormone modulation of genes on the X chromosome that modify epigenetic profiles in susceptible women. Two observations demand explanation in understanding the genetic predisposition to SLE. Firstly, there is little concordance between monozygotic twins, and secondly the F:M ratio of neonates with SLE is approximately 1. These observations imply *in utero* environments affecting both sexes equally predispose to the development of SLE. The obvious candidate is temporal differences in the *in utero* sex hormonal environment during pregnancy. That this environmental factor is relevant is supported by both abnormal androgen levels during early pregnancy of mothers with SLE and especially by the decreased 2D4D ratios reported in both males and females with SLE. Given the consistent variation of 2D4D ratios in persons with many types of traits that exhibit gender imbalance, temporal *in utero* androgen imbalance seems to be an important feature of SLE. The dramatic increase in SLE incidence during the reproductive years in women may reflect a synergistic effect of increased estrogen levels in females predisposed during pregnancy to androgen imbalance.

There is much evidence to support the hypothesis that SLE arises from defects in apoptosis - the complex physiological mechanism whereby the detritus of dead and damaged cells is removed from the body following programmed cell death (17, 18). Relatively rare protein deficiency states mediated by autosomal single locus mutant genes are closely associated with SLE as described above. These loci occur in equal frequency in males and females and SLE is often inherited in kindreds in Mendelian fashion. They are examples of rare genes of major predisposition for SLE. The evidence to date is that in a minority of cases the rare deficiency state occurs in persons who not develop SLE as children but may do so later in life. A conservative estimate of the proportion of SLE cases that may be accounted for by this type of deficiency state is $\approx 5\%$ of children within the first decade of life^(a).

(a) The assumption is made based on our own meta-analysis.

1.15 Tables and figures

1.15.1 Tables

Table 1.1: Meta-analysis of Incidence and Prevalence of SLE per 100,000 person years from 4 seminal studies and 1 Australian study.

Data Source	Gender	Caucasians		Black		Overall	
		Incidence	Prevalence	Incidence	Prevalence	Incidence	Prevalence
(24)	Females	6.3	86.7	12.8	186.3	9.3	127.8
	Males	1.2	8.7	2.1	19.3	1.6	12.4
	F:M ratio	5.3	10.0	6.1	9.7	6	10
(23)	Females	4.7	59	13.4	196.2	9.4	131.1
	Males	0.7	7.5	3.2	23.7	1.7	14.9
	F:M ratio	6.7	7.9	4.2	8.3	6	9
(26)	Females					5.51	79.1
	Males					0.92	11.83
	F:M ratio					6.0	6.7
(25)	M & F	6.73	134.53	22.5*	247.62*	8.34	167.62
						1.44	24.82
	F:M ratio					5.8	6.8
(27)			30.2**		92.8		45.3
(Aust. Indigenous)	F:M ratio		6.5**		5.5		6.2

*Average from Black African, Caribbean & other. ** Estimated from data included in paper. Grey shaded boxes represent non-available or not applicable data.

Table 1.2: SLE concordance/discordance in Human twin

References	Gender	Monozygotic Discordance	Dizygotic Discordance	Monozygotic Concordance	Dizygotic Concordance	Total Monozygotic sets	Total Dizygotic sets
(173)	F	1	n/a	0	n/a	1	n/a
(44)	All F, 1 M	6	0	13	1	19	1
(174)	F	3	3	3	0	6	3
(175)	M	1	n/a	0	n/a	1	n/a
(176)	F	1	n/a	0	n/a	1	n/a
(177)	M	1	n/a	0	n/a	1	n/a
(48)	F	1	n/a	0	n/a	1	n/a
(178)	1 MZ M	8	10	1	0	9	10
(179)	F & 1 DZ M co-twin	3	6	1	0	4	6
(180)	F, 1M	9	n/a	0	n/a	9	n/a
(181)	F	4	n/a	1	n/a	5	n/a
(47)	F	0	n/a	1	n/a	1	n/a
(182)	F	1	n/a	0	n/a	1	n/a
(45)	M	0	n/a	1	n/a	1	n/a
(49)	F	1	n/a	0	n/a	1	n/a
(183)	F, 1 DZ M	6	4	0	0	6	4
(184)	F	1	n/a	0	n/a	1	n/a
(185)	F	1	n/a	0	n/a	1	n/a
(46)	F	0	n/a	1	n/a	1	n/a
Total		48	23	22	1	70	24
%		68.6%	95.8%	31.4%	4.2%	74.5%	25.5%
Discordance Total		75.5%					
Concordance total		24.5%					

Table 1.2 shows data from 19 studies published between 1975 and 2013 that included twin sets, monozygotic or dizygotic in which either one or both the siblings had SLE. Grey shaded boxes represent non-available or not applicable data. The gender column shows the gender of the probands: F – females, M – Males, MZ – monozygotic, DZ – dizygotic. If the gender status was not clear in the original studies, it was assumed that female twin sets were included.

Table 1.3: Summary of neonatal births with SLE from 2013 - 2016

Study	Neonate gender	Neonate ethnicity	Maternal history
(186)	7 males & 8 Females	8 Caucasians, 4 individuals from African descent, 2 Asians, 1 unknown	Connective tissue disorder in 1 mother
(187)	Female	Caucasian	
(188)	Male	Asian	Sjögren syndrome
(189)	Male	Caucasian (?)	
(190)	Female		
(191)	Male	Arab	Active rheumatoid Arthritis
(192)	Female	Asian	
(193)	Male	Caucasian	Antibodies positive*
(194)	Female		SLE
	Male	Black	None
	Female		Juvenile idiopathic arthritis
	Female		None
(195)	5 Females & 7 males		
(196)	2 Males, brothers (diagnosed 2 years apart)		Antibodies positive*
(197)	Male	Senegalese	
(198)	Male		
(199)	Female		
(200)	34 females out of 58		History systemic sclerosis, SLE or both in some
(31)	6 Females 1 male (with inherited complement deficiency)	Arabs	
(201)	Female	Asian American	
(202)	Male	Caucasian	
	Female	South American	
	Female	Caucasian	
	Male	Caucasian	Sjögren syndrome
(203)	Male		Sjögren syndrome

Antibodies positive* indicates that the mother was recorded with positive levels of anti RO/SM antibodies. This table shows data extracted from 19 recent studies reporting on cases of neonatal lupus. Studies included the proband's gender, their ethnicity and their mother's medical history at the time the neonate was diagnosed. Shaded boxes indicate cases where no data was available.

Table 1.4: Counting expected SLE cases in a Caucasian population of one million persons (M:F =1)

Deficiency genotype)	Chromosome	Prevalence x 10 ⁻⁶	Concordant with SLE %	Sibling SLE concordance %	Female SLE	Male SLE	F:M ratio concordant siblings**	References
Population	Caucasoid	811	N/A	N/A	730	81	9	This review
	Black	2125			1910	215	9	
C1q	1p36	1	93	90	0.5	0.5	1.3 ≈ 1	(2)
C1r and C1s	12p13	1	57	67	0.5	0.5	1.7	(2)
C2	6p21	400 or 100 G	10	58	8.75	1.25	7:1	(2)
Total C4	6p21	1	75	80	0.7	0.3	2.1 ≈ 2	(2)
(C4A + C4B)								
FeGR Complex	1q23	10	100	N/A	5	5	1	(4, 5)
TREX1#	3p21.31	20	100	2	10	10	1	(6, 7)
(DNASE III exonuclease)								
Extracellular DNASE I & II	≈3p14.3	1	75		0.5	0.5	1	(8, 9)
Kleinfelter's syndrome	Karyotype XXY	1700 [^]	2.5	N/A	0	1.0	N/A	(10, 11)
Triple X syndrome	Karyotype XXX	1000 [^]	4	N/A	4	0	N/A	(12)
No. predicted					30	19	1.6	
% Predicted					4%	23%	0.17	
					1.6%	10%	0.16	

* For rare deficiency states with no reliable published incidence/prevalence data, the prevalence has been assumed to be of the order of 1 x 10⁻⁶, includes complete deficiency and heterozygous deficiencies. ^ Prevalence per 500K females or males only. Grey shaded boxes and N/A represent non-available or not applicable data.

1.15.2 Figures

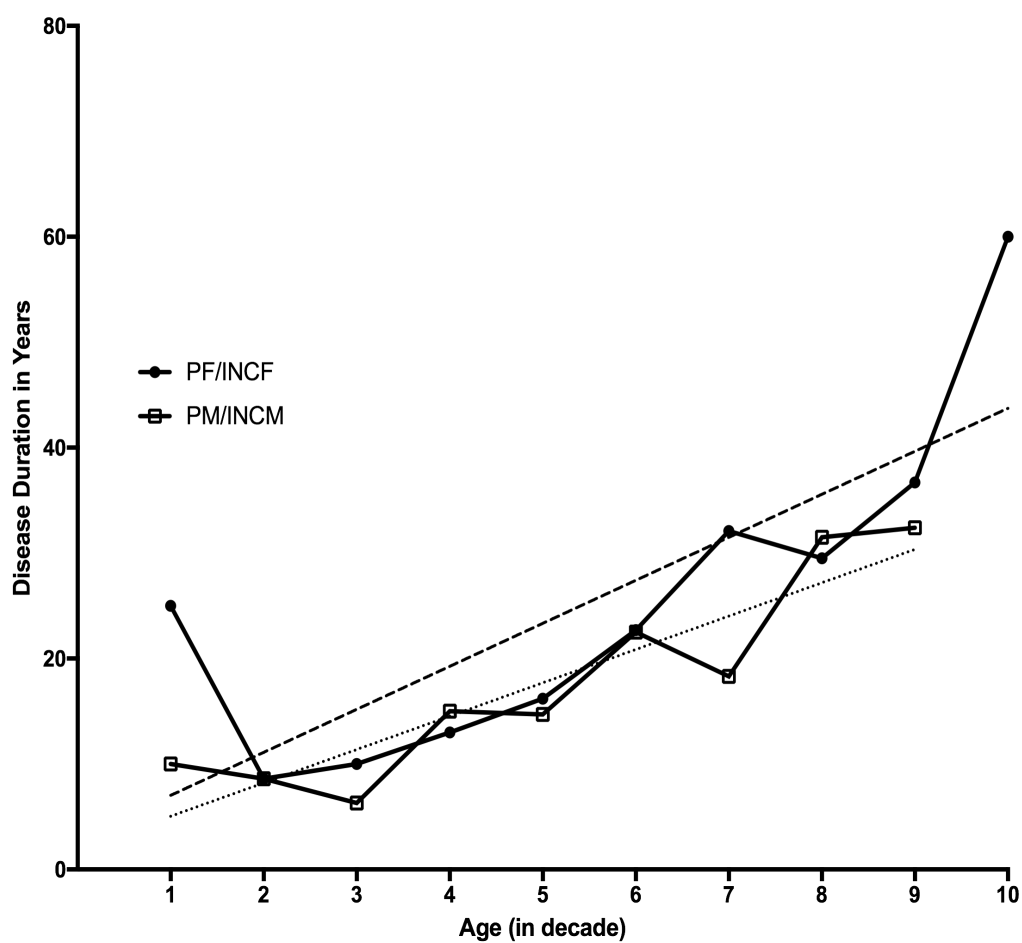


Figure 1.1: Duration of SLE per decade estimated from age based prevalence to incidence ratio in years.
Estimates derived from the data of Rees (25, 28).

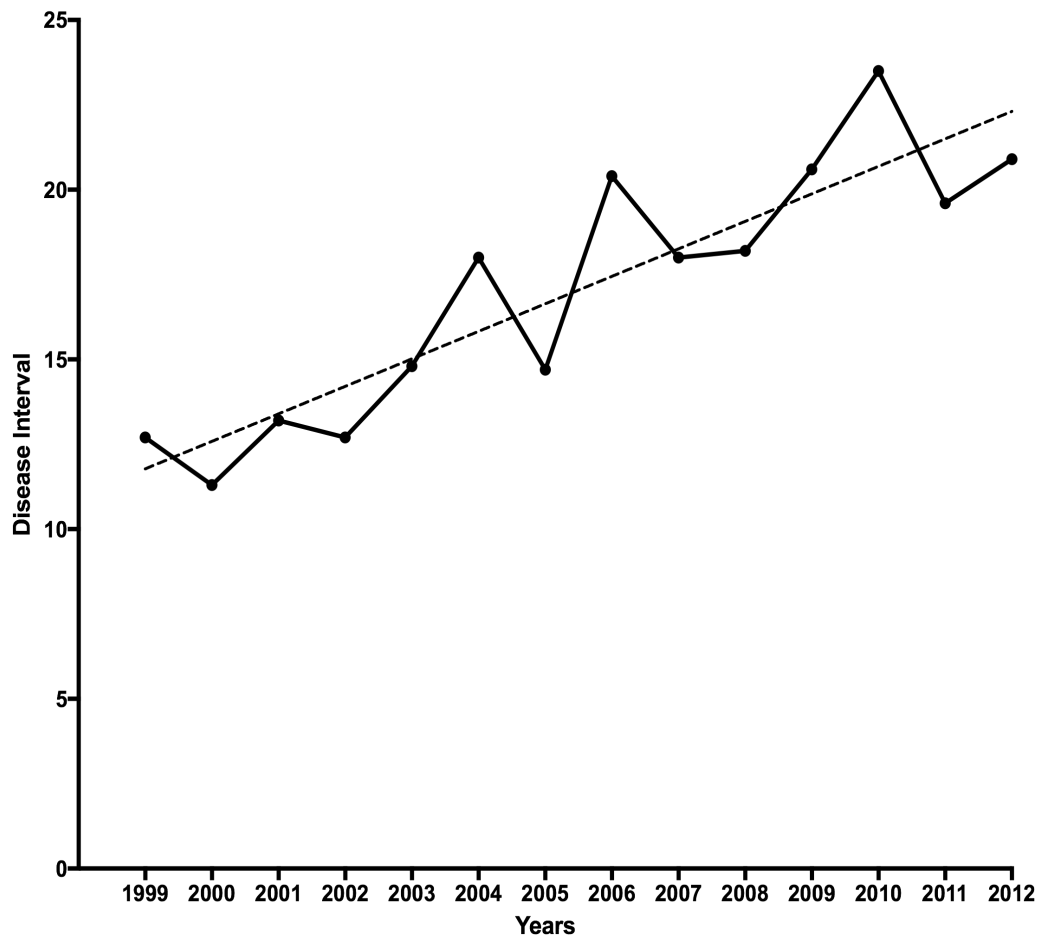


Figure 1.2: Estimated duration of SLE in a mixed UK population per calendar year.
Estimated from the data of Rees (25). Disease duration(years) = prevalence/incidence per year.

Chapter 2. Material and methods

2.1 Human ethics approval

Curtin University, Sir Charles Gairdner Hospital and Royal Perth Hospital Human Ethics Committees approved the human studies (approval numbers HR 202/2013, HREC 2013-174). Written informed consent was obtained from all study participants. Individuals under 18 were excluded from participation.

2.2 Patient cohort

Fifty-six (56) treated systemic lupus erythematosus patients aged 47 ± 15 years (91% females) were recruited from Sir Charles Gairdner (SCGH) and Royal Perth Hospitals (RPH - Perth, Western Australia) between March 2014 and December 2015. Diagnoses were based on the American College of Rheumatology (ACR) classification criteria. Aside from two patients with active disease, this population of SLE patients was well-managed, with no evidence of disease activity based on laboratory parameters and/or clinical examination.

In addition to the SLE diagnosis, patients had co-morbidities including renal disease, liver pathologies as well as Sjögren and Raynaud's syndromes. SLE patients in this cohort were treated with various therapeutic regimens including steroids (low dose prednisolone ($\leq 5\text{mg/day}$)), steroid-sparing immunosuppressant therapies (azathioprine, cyclosporine, cyclophosphamide, methotrexate, mycophenolate mofetil) and antimalarial therapy (hydroxychloroquine), summarised in Table 2.1.

2.3 Healthy controls cohort

Thirty-three age and sex-matched healthy control subjects (47 ± 15 years – 80% females) were recruited from the Perth metropolitan area.

2.4 Sample collection and preparation

Peripheral blood samples from SLE patients were collected at one time point during the clinical management of this cohort of pre-diagnosed SLE patients. Blood was collected into ethylenediaminetetraacetic acid (EDTA), heparin and serum collection tubes at PathWest collection centres. Serum samples were separated by centrifugation at PathWest laboratories within one hour of collection and frozen to between -70°C and -86°C. Samples from healthy human controls were collected into EDTA, heparin and serum vacutainer tubes (Beckton Dickinson (BD), San Jose, USA) and processed in the same manner at Curtin University laboratory (Perth, WA).

Peripheral blood mononuclear cells (PBMCs) were isolated by diluting the remaining fraction one in two in phosphate buffered saline (PBS; Sigma-Aldrich, Sydney, Australia), followed by density gradient centrifugation on Ficoll-Paque™ Plus (GE Healthcare, Little Chalfont, United Kingdom). PBMCs were washed three times in PBS and re-suspended in RPMI 1640 (Invitrogen, California, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Scoresby, Australia), PenStrep (at final concentrations of 100 units/ml penicillin and 100 µg/ml streptomycin; GE Healthcare), 2 mM L-glutamax (Invitrogen) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich).

2.5 Flow Cytometry

2.5.1 Cell subsets identification

PBMCs were stained with fluorescently-labelled mouse antibodies directed against human molecules listed in Table 2.2.

2.5.1.1 Identification of dendritic cell subsets

Identification of dendritic cell (DCs) subsets first involved use of an exclusion cocktail consisting of antibodies directed against lineage markers CD3 (T cells), CD14 (monocytes), CD19 (B cells), CD16 (Fc receptor⁺ cells), CD20 (B cells) and CD56

(natural killer cells). Visual graphic representation of the gating strategy for dendritic cell subsets is shown in Figure 2.2.

Dendritic cell subsets sitting located in the human lineage cocktail negative gate were identified as:

- Myeloid dendritic cells type 1: mDC1s (CD1c⁺CD123⁻CD303⁻)
- Myeloid dendritic cells type 2: mDC2s (CD141⁺CD123⁻CD303⁻)
- Plasmacytoid dendritic cells: pDCs (CD1c⁻CD141⁻CD123⁺CD303⁺)

2.5.1.2 Identification of T cell subsets

Visual graphic representation of the gating strategies for helper T cell subsets and regulatory T cells is shown in Figure 2.3 and Figure 2.4. T cell subsets were identified as:

- Gamma delta T cells (TCR $\alpha\beta$ ⁻ TCR $\gamma\delta$ ⁺)
- Regulatory T cells: Tregs (CD3⁺CD4⁺CD25⁺CD127^{low/neg})
- T Helper cells 17: Th17 cells (CD3⁺CD4⁺CD161⁺CD196⁺)

2.5.1.3 Gating strategies

For all staining panels, a combination of isotype controls and single stains were used for compensation and determination of gates. Viable cells were identified using Zombie Green™ Fixable Viability Kit (Biolegend, USA). Analysis was performed on a FACSCanto II using FACSDiva software (BD) or FlowJo software (TreeStar, Oregon, USA).

2.5.2 Staining Procedure

2.5.2.1 Dendritic cells

PBMCs were incubated with antibodies for 30 minutes at 4°C in the dark, washed using PBS and re-suspended in 200 µl of fluorescence activated cell sorting (FACS) buffer (1x PBS/1% FBS/1% bovine serum albumin).

2.5.2.2 T cells

For intracellular cytokine staining (Treg, $\gamma\delta$ T cells and Th17 cells) PBMCs were incubated with 10 µg/ml brefeldin A (Sigma-Aldrich) for four hours in the dark at 37°C in a 5% CO₂ atmosphere to prevent protein movement from the endoplasmic reticulum to the golgi apparatus and inhibit cytokine secretion; centrifuged, washed in FACS buffer containing 2.5 µg/ml brefeldin A and then incubated with antibodies directed against cell surface molecules for 30 minutes at 4°C in the dark, before being washed twice in FACS buffer containing 2.5 µg/ml brefeldin A, and fixed with 2% paraformaldehyde (Sigma-Aldrich)/PBS for 15 minutes at 4°C in the dark. After fixation cells were washed twice in FACS buffer and incubated in FACS buffer containing 0.1% saponin (Sigma-Aldrich) for 15 minutes at 4°C in the dark to allow target antibodies to penetrate cells, after which the cells were stained with anti-IL-17 antibody diluted in FACS buffer containing 0.1% saponin, then washed twice in FACS buffer and re-suspended in 200 µl FACS buffer.

2.5.3 Cytokine measurement

Interleukin 1 beta (IL1 β), interferon alpha (IFN α), interferon gamma (IFN γ), tumour necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1/ CCL2), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12p70), interleukin 17A (IL-17), interleukin 18 (IL-18), interleukin 23 (IL-23) and interleukin 33 (IL-33) concentrations in serum were measured using a human LEGENDplex™ cytometric bead array (CBA; Biolegend) according to the manufacturer's instructions. Briefly, samples and cytokine standards were incubated with cytokine capture beads in a 96-well plate for two hours, followed by incubation

with phycoerythrin (PE)-conjugated detection antibodies for 30 minutes, both at room temperature, in the dark and on a plate shaker at approximately 600 rpm. Incubation was followed by washing with 200 µl of 1x wash buffer per well and centrifugation at 1000 x *g* for five minutes. Samples/standards were re-suspended in 200 µl of wash buffer and analysis performed on a FACS Canto II using FACS Diva software (BD).

2.6 Serology

2.6.1 Complement C1q, C3, C4 concentrations, C-reactive protein, double stranded DNA antibodies assays

Patients data for dsDNA ab, C-reactive protein (CRP), C4 and C3 were collected from the patients' files and corresponded to the time-point at which they were recruited and/or blood samples obtained for the study. All the above parameters were assayed in the PathWest Diagnostic Laboratories at Royal Perth Hospital and Sir Charles Gairdner Hospital. Samples tested for antibodies to double stranded DNA: (dsDNA ab) were assayed using a radioimmunoassay dsDNA ab kit (Trinity Biotech; NY, USA) as per the manufacturer's directions. C-reactive protein (CRP) concentrations in serum or plasma samples were assessed using the turbidometric anti-CRP antibody coated latex particle assay in an Abbott Architect C16000 instrument (Abbott Laboratories, Abbott Park, Illinois USA). Complement protein C3 and C4 concentrations in serum or plasma samples were also measured by turbidometry assay using the Abbott Architect C16000 instrument. Complement C1q levels were assayed using a nephelometry method on a Siemens BN2 nephelometer.

2.6.2 αC1q ab concentrations

A solid-phase ELISA kit (Bulhmann Laboratories, Schönenbuch Switzerland) was used to quantify IgG αC1q ab specific for the neo-antigen generated on solid phase C1q. Stored patient sera were diluted in high salt buffer (0.5M NaCl) and incubated in microtiter wells coated with human C1q. Horseradish peroxidase (HRP)- labelled anti-human IgG was then added, followed by tetramethylbenzidine (TMB) substrate.

A washing step was included between each incubation. The substrate reaction was terminated through the addition 0.25M sulphuric acid and the absorbance measured at 450 nm using a microtiter plate reader (Bio-Rad, California, USA). Test sample concentrations were interpolated using a four-parameter logistic calibration curve fitted to the standards provided by the manufacturer (5, 25, 100 and 400 U/mL based on an international reference standard). The manufacturer's suggested value for positivity was 15 units/ml.

2.6.3 DNASE I enzyme concentrations

A sandwich ELISA kit (Creative Diagnostics, USA) was used to assay human DNASE I serum concentrations per manufacturer's instructions. Briefly, stored patient sera were incubated in microtiter wells coated with a DNASE I specific antibody. DNASE I present in the samples was immobilized and unbound products removed. Biotin labelled DNASE I specific antibody bound to immobilized DNASE I was detected with avidin conjugated Horseradish Peroxidase (HRP). Test sample concentrations were interpolated using a four-parameter logistic calibration curve fitted to the standard sera provided by the manufacturer (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0ng/mL).

2.6.4 Serum C4 concentrations normal ranges

We previously defined normal serum C4 mean concentrations and ranges for two, three, four and five total C4 gene copy numbers (GCN) respectively (86).

These ranges were as follow:

- Two GCN: mean 0.197; 95% confidence intervals 0.059 – 0.343
- Three GCN: mean 0.256; 95% confidence intervals 0.117 – 0.383
- Four GCN: mean 0.296; 95% confidence intervals 0.159 – 0.439
- Five GCN: mean 0.425; 95% confidence intervals 0.279 – 0.571

2.7 Molecular assays

2.7.1 Determination of complement C4, C4A and C4B gene copy number, HERV-K retroviral element in intron 9 and CT insertion in exon 29 of the C4 genes

2.7.1.1 Complement C4 genes and HERV-K retroviral element in intron nine of the C4 genes

The complement C4 gene copy number was assessed using Taqman™ real-time PCR methodology which was previously described (86). The presence of the HERV-K retroviral element in intron nine of the C4 genes was assessed by real-time PCR on a VIIA 7 thermocycler (ThermoFisher Scientific) using Taqman™ (Life Technologies, California; USA) custom copy number assay designed from published primers (204) (see Table 2.3) and performed according to the manufacturer's instructions in 96-wells plates, with total volume 20µl per reaction containing:

- 4µl of test DNA
- 10µl of 2X TaqMan Genotyping Master Mix
- 1µl of 20X TaqMan C4 - long (C4L) or TaqMan C4 - short (C4S) custom GCN assay
- 1µl of 20x TaqMan copy number reference assay (RNase P component H1)
- 4µl of nuclease free water

PCR assays were performed with cycle parameters set at 95°C for 10 minutes and 40 cycles consisting of 95°C for 15 seconds followed by 60°C for 60 seconds. In each plate, controls were added which consisted of Major Histocompatibility Complex (MHC) samples (cell lines WT100BIS, MOU, SAVC, JESTHOM, HOM-2, CBG-IB CB6B kindly donated by the Immunology department at Fiona Stanley Hospital; Perth, Western Australia).

The CT insertion in exon 29 was detected by sequencing after PCR amplification in PCR microfuge tubes with total volume 50µl per reaction as follow:

- 4µl of test DNA
- 10µl of 5x My Taq reaction buffer (Bioline, USA)
- 1µl each of the forward and reverse primers 20µM
- 0.5µl of My Taq HS DNA polymerase (Bioline, USA)
- 33.5µl of nuclease free water

PCR assays were performed with cycle parameters set at 95°C for 1 minutes (1 cycle) and 35 cycles consisting of 95°C for 15 seconds followed by 53°C for 15 seconds with a product size of 770bp. The primers sequences are available in Table 2.3. The PCR products were then sent for sequencing (MACROGEN, Seoul; Korea) and the sequences were assessed for the presence of the CT insertion.

2.7.2 Identification of phenotypes and genotypes at the DNASE I and intron 4 VNTR (HumDN1) loci.

Stored DNA samples were used in a PCR reaction using MyFi™ DNA polymerase (Bioline, USA) to amplify exons (or part of) two, five, six, seven and eight using primers sets described in Table 2.4.

The PCR amplifications were performed in total volume of 25µl per reaction in PCR microfuge tubes containing:

- 1µl of test DNA
- 5µl of 5x MyFi reaction buffer (Bioline, USA)
- 0.5µl of each forward and reverse primers 10µM
- 1µl of MyFi DNA polymerase (Bioline, USA)
- 17µl of nuclease free water

PCR assays were performed with cycle parameters set at 95°C for 1 minutes (1 cycle) and 35 cycles consisting of 95°C for 15 seconds followed by 61°C for 15 seconds (DNASE I) or 57°C for 15 seconds (VNTR – *HumDN1*).

PCR products were then then sequenced (MACROGEN) to identify exonic alleles of Human DNASE I as indicated in Table 6.1. The number of VNTR (*HumDN1*) repeats within intron four of the Human DNASE I gene was determined using a PCR reaction with MyFi™ DNA polymerase (Bioline, USA) to amplify intron four spanned by exons four and five using the primers pair shown in Table 2.4. The lengths of the amplicons were then estimated via electrophoresis on a 4% agarose gel using a 1kb DNA ladder (see Figure 2.1). As a control measure, five samples were sent for sequencing (details as above) and the results compared with the gels. Alleles manifested three, four, five and six repeats with corresponding molecular weights of 525, 581, 637 and 693bp respectively (205).

2.8 Data analysis

Statistical significance was calculated using Mann–Whitney ranking *U*-test with the program GraphPad PRISM 7 (GraphPad Software Inc, California, USA). *P* values of < 0.05 were considered statistically significant.

2.8.1 Cellular assays

Cell subsets and their expressed cytokines' raw data were transformed using $\log_{10}(x + 1)$ before calculating statistical significance using Mann–Whitney ranking *U*-test on the program GraphPad PRISM 7 (GraphPad Software Inc, California, USA). *P* values of < 0.05 were considered statistically significant, but only values <0.001 were interpreted as experimentally significant after allowing for Bonferroni correction

2.8.2 Molecular assays

Gene copy number variations were analysed using CopyCaller® version 2 (Life Technologies, California; USA) and GraphPad PRISM 7 software (GraphPad Software Inc, California, USA). Nucleotide and peptide sequences were analysed using Geneious software version 10 (206). Linkage disequilibrium and population parameters were determined using “Genepop on the web” (207, 208), LinkDos (209) and SNPStats (210). Statistical significance was calculated using Mann–Whitney rank

sum *U*-test with the GraphPad PRISM 7 application (GraphPad Software Inc, California, USA). *P* values of < 0.05 were considered statistically significant.

2.9 Reference tables

Table 2.1: SLE patients co-morbidities and treatment regimen data.

	Steroids (1)	Anti- malarial (2)	Immuno- suppressants (3)	1 + 2	1 + 3	2 + 3	Combination of 1, 2 & 3	Other therapeutics
Renal symptoms (N=11)	8 (72.7%)	7 (63.6%)	8 (72.7%)	1 (9.1%)	3 (27.3%)	0 (0%)	4 (36.4%)	8 (72.7%)
Hepatic involvement (N=4)	2 (50%)	4 (100%)	2 (50%)	1 (25%)	0 (0%)	1 (25%)	1 (25%)	3 (75%)
Anti-phospholipid (N=17)	13 (76.5%)	9 (52.9%)	7 (41.2%)	3 (17.6%)	3 (17.6%)	0 (0%)	4 (23.5%)	16 (94.1%)
Raynaud's phenomenon (N=4)	2 (50%)	2 (50%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	4 (100%)
Sjögren's disease (N=4)	0 (0%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (75%)
Cutaneous/discoid symptoms (N=12)	5 (41.7%)	6 (50%)	7 (58.3%)	1 (8.3%)	0 (0%)	0 (0%)	3 (25%)	9 (75%)
Neuronal symptoms (N=1)	1 (100%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
T1DM (N=3)	3 (100%)	2 (66.7%)	2 (66.7%)	1 (33.3%)	1 (33.3%)	0 (0%)	1 (33.3%)	3 (100%)
Other symptoms (N=13)	9 (69.2%)	11 (84.6%)	7 (53.8%)	2 (15.4%)	2 (15.4%)	1 (7.7%)	4 (30.8%)	13 (100%)

The table indicates the number and percentage of patients in each category of lupus manifestation under specific types of treatments (Patients may fit into multiple lupus manifestations as well as treatments under review with other type of medications).

Table 2.2: List of antibodies used to detect surface and intracellular markers via flow cytometry

Cell markers (all mouse anti-human antibodies)	Clone	Fluorochrome
CD3 (T cells)	UCHT1	
CD14 (monocytes)	HCD14	
CD19 (B cells)	3G8	
Lineage Negative		
CD16 (Fc receptor+ cells)	HIB19	Fluorescein isothiocyanate (211) (211)
CD20 (B cells)	2H7	
CD56 (natural killer cells)	HCD56	
CD1c (BDCA)	L161	Alexa Fluor® 647 (AF647)
CD141 (BDCA3/thrombomodulin)	M80	Brilliant Violet 421™ (BV421)
CD123 (BDCA2)	6H6	PerCP-Cyanine5.5 (PerCP-Cy5.5)
CD303 (BDCA3)	201A	PerCP-Cy5.5
α/β TCR	IP26	FITC
γ/δ TCR	B1	Brilliant Violet 510™
CD3	SK7	Streptavidin-Allophycocyanine H7 (APC-H7)
CD4	OKT4	Phycoerythrin Cyanine 7, (PE/Cy7)
CD25	M-A251	Brilliant Violet 510™ (BV510)
CD127 (IL-7R α)	A019D5	Alexa Fluor® 488 (AD488)
CD161 (killer cell lectin-like receptor subfamily B member 1 or KLRB1, also known as NK1.1)	HP-3G10	BV510
CD196 (chemokine receptor 6 or CCR6)	G034E3	AF488
IL-17A	BL168	BV421
Latency-associated peptide (LAP; TGF β)	TW4-2F8	PerCP-Cy5.5

All antibodies were purchased from Biolegend (San Diego, California; US) apart from CD3 which was purchased from BD Biosciences (212).

Table 2.3: Primers used to identify the presence/absence of the HERV-K retroviral element in intron 9 and the CT insertion in exon 29 of C4 genes.

Name	Forward primer (5' – 3')	Reverse primer (5' – 3')
C4 long (with HERV-K insertion)	TTGCTCGTTCTGCTCATTCCTT (C4L: C4Fin95)	GTTGAGGCTGGTCCCCAACA (C4L-3LTR-R)
C4 short (without HERV-K insertion)	TTGCTCGTTCTGCTCATTCCTT (C4S: C4Fin95)	GGCGCAGGCTGCTGTATT (C4Sin9R-2)
Probe C4in95 FAM/MGB	CTC CTC CAG TGG ACA TG	
TC insertion	TTG CCC ACA ACA ACCT CA TG	CCT ACT TGG GTA CTG CGG AA

Primers from GeneWorks.

Table 2.4: Primers used to identify common alleles of the DNASE I gene and the 56bp VNTR (HumDN1) repeat.

Genic Location	Forward primer (5' – 3')	Reverse primer (5' – 3')
Exon 2	TAT GTC TCT GTG CCC TGT GC	CAG AGC TCC TCT GCT TTT GG
Exons 5 & 6	CTG GGG TCA CCT CCT CCT	AGC GCT GAT CTC GGC TAC T
Exons 7 & 8	CCT GGT AGG ACG TCA TGT TG	TGT GCA AGG GAG GAC ACA TA
VNTR (HUMDN1) Ref: (205)	GGA CCT TTT GTT TCT TCA A	ACC GCA GAC ACC TGG TCA

2.10 Figures

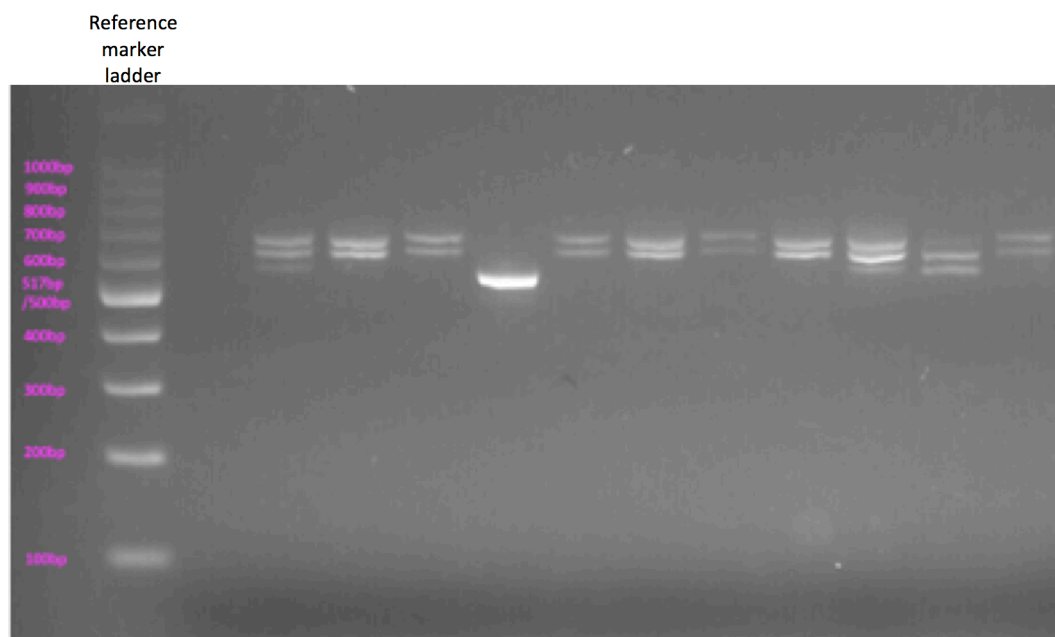


Figure 2.1: Agarose electrophoresis gels representative of VNTR sizes.

The figure shows a representative example of a 4% agarose electrophoresis gel used to assess the size of the VNTR (*humDN1*) repeats.

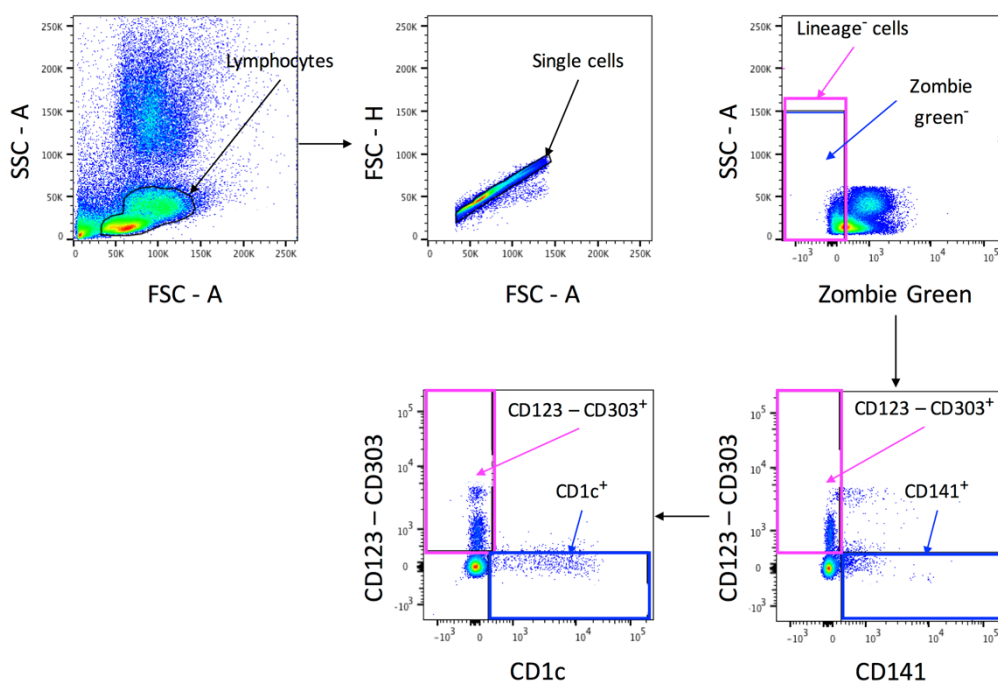


Figure 2.2: Dendritic cells gating strategy.

Figure 2.2 shows the gating strategy to identify dendritic cell subsets as described above.

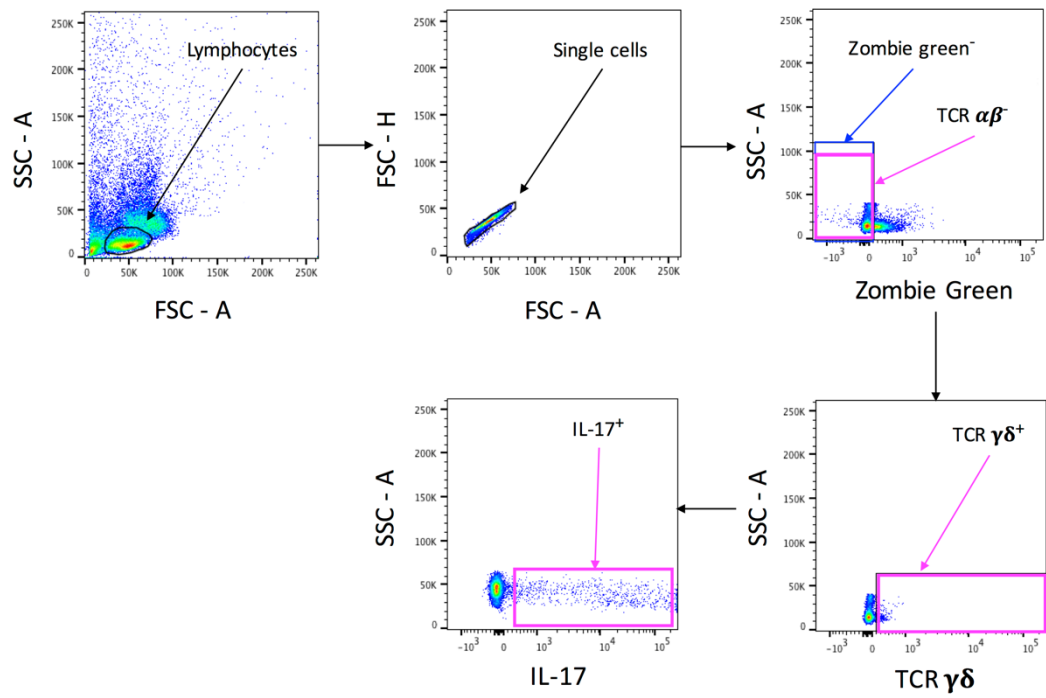


Figure 2.3: Gamma Delta T cells gating strategy.

The figure shows the gating strategy to identify gamma-delta T cells as described in the text.

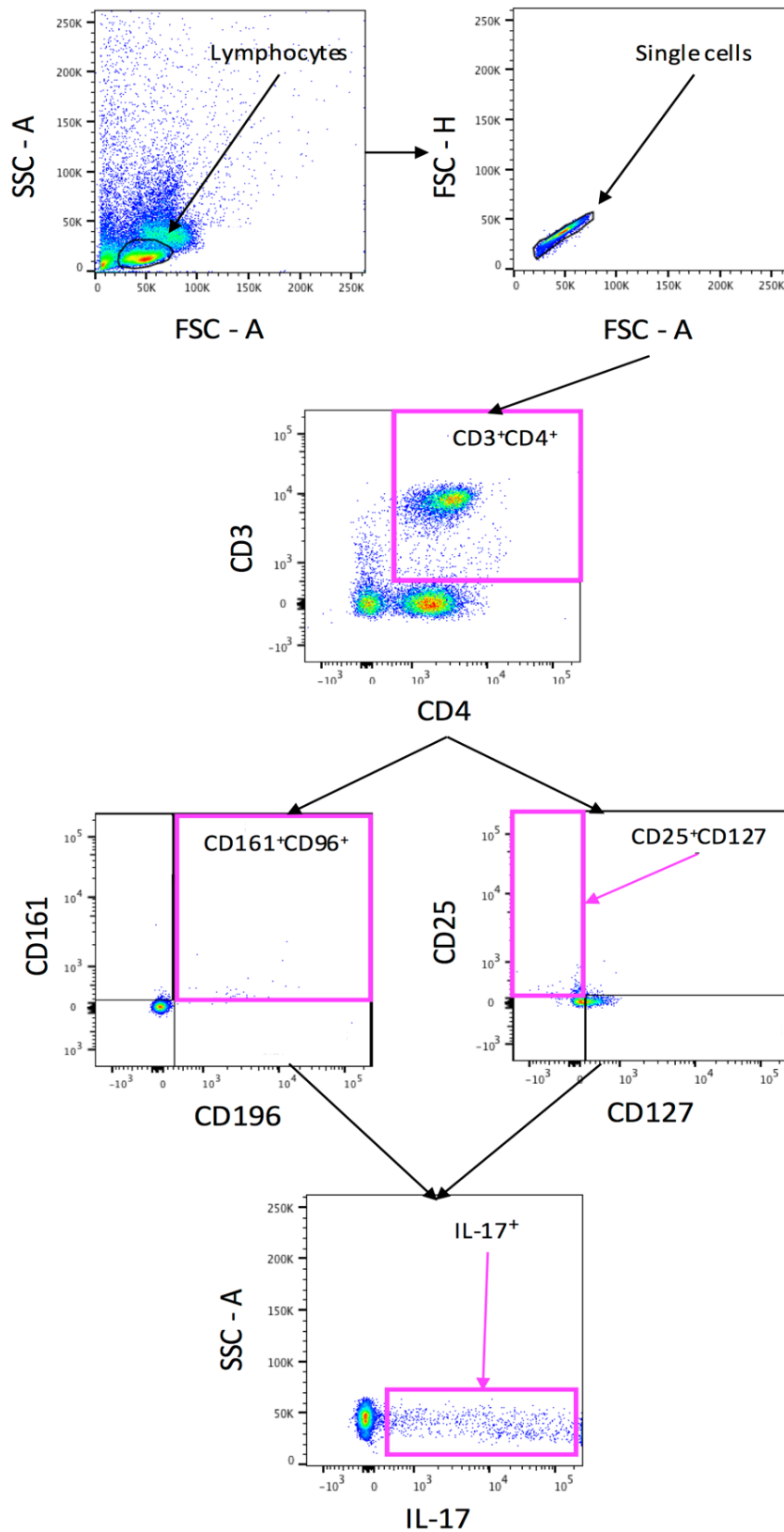


Figure 2.4: T regs & Th17 cell gating strategy.

The figure shows the gating strategy to identify regulatory T cells and Th17 cells as described in the text.

Chapter 3. Elevated circulating myeloid dendritic-2 cells and gamma delta T cell-derived interleukin-17 contribute to inflammation in systemic lupus erythematosus

3.1 Abstract

Objectives: Cellular profiles and inflammatory cytokines were assayed in fresh blood samples from 56 SLE patients and 33 age/sex-matched controls.

Methods: Myeloid dendritic cell (mDCs) 1 and 2, plasmacytoid DCs (pDCs), regulatory T cells (Tregs), gamma delta T cells ($\gamma\delta$ T cells) and T helper 17 (Th17) cell frequencies were assayed by flow cytometry, together with the synthesis of intracellular interleukin 17 (IL17) and transforming growth factor beta (TGF β). Additionally, a panel of 13 inflammatory cytokines were assayed by cytokine bead array in serum samples.

Results: mDC1 and mDC2 proportions were decreased in SLE patients (0.1096 & 0.1774 respectively) relative to healthy controls (0.2471 & 0.2320 respectively - $p < 0.0001$ and $p = 0.0121$ respectively). The proportions of Tregs and Th17 cell populations were similar in both groups. In contrast, the proportion of $\gamma\delta$ T cells was increased in SLE patients relative to controls, although the difference did not reach statistical significance. Serum concentrations of 12 of the 13 inflammatory cytokines appeared elevated in SLE patients, with six cytokines attaining a p value ≤ 0.05 (IFN γ TNF α , MCP-1, IL-6, IL-8 and IL-18; $p = 0.0193, 0.0317, 0.0002, 0.0190, 0.0350$ and < 0.0001 respectively). Intracellular synthesis of IL-17 occurred predominantly in $\gamma\delta$ T cells, and concentrations were significantly higher in patients compared to healthy controls ($p = 0.0010$). A correlation matrix revealed associations between $\gamma\delta$ intracellular IL17 synthesis and $\gamma\delta$ T cell subsets ($p < 0.0001$) and between mDC2 and $\gamma\delta$ T cell subsets ($p = 0.0408$) in SLE.

In summary, despite literature demonstrating an increased frequency of Th17 cells and elevated intracellular IL17 in other SLE cohorts, this was not observed in this SLE cohort. The relative increase in $\gamma\delta$ T cells and mDC2 cells proportions is a novel observation and represents a potential contributor to inflammation in SLE that requires further investigation.

3.2 Introduction

Systemic lupus erythematosus (SLE), a polygenic, multifactorial autoimmune disease, is characterised by self-antigen/ autoantibody production, immune complexes and chronic inflammation leading to multiple organ and tissue damage (213). Recent studies have shown that impaired apoptosis leads to generation of immune complexes and aberrant inflammation (213).

Dendritic cells (DCs) can be classified as myeloid (mDCs) and plasmacytoid (pDCs) and are involved in the preservation of tolerance in the healthy steady state (214). Apoptotic micro-molecules present in SLE patients have been shown to activate mDCs and pDCs, promoting a pro-inflammatory response (17), and apoptotic materials ingested by DCs from healthy mice stimulate CD4⁺ T helper 1 (Th1) and T helper 17 (Th17) cell polarisation (215). The mDC1 subset synthesises a variety of inflammatory chemokines and cytokines and are the most abundant DC subpopulation, whilst mDC2 cells are the rarest in peripheral blood from healthy humans (216, 217) that can cross-present antigens to cytotoxic CD8 T cells, synthesise interleukin (IL) 12 (218) and IFN γ (219). These cells also synthesise high amounts of IL-12p70 upon activation by toll-like receptors (TLRs) and CD40 molecules (220). Active SLE patients have significantly lower numbers of peripheral blood DCs yet they retain adequate and similar functionality (221). DCs cross talk with other cell types such as gamma delta T cells ($\gamma\delta$ T cells) to amplify immune responses (222). Moreover, bacterial lipopolysaccharide can activate DCs and $\gamma\delta$ T cells, and $\gamma\delta$ T cells reciprocate by enhancing maturation of activated DCs (223).

Few studies have investigated the role of $\gamma\delta$ T cells in the pathogenesis of lupus, and those that have are contradictory. Peripheral blood $\gamma\delta$ T cell frequencies have been reported to be increased (224), decreased (225-228) or unchanged (229) in SLE patients. Likewise, regulatory T cells (Tregs), a suppressive subset that expresses CD4, the transcription factor forkhead box P3 (Foxp3) and the interleukin 2 receptor (IL-2R/ CD25) (230) also demonstrate decreased (230-236), increased (237), or unchanged (238-241) frequencies in SLE patients, while positively (242-246) or inversely correlating (232) with disease activity. Th17 cells produce IL-17A, IL-17F, IL-

22 and IL-21 (247-250) and are a potent source of inflammation by mechanisms that include enhancement of neutrophil and macrophage activation and function (249, 250). A combination of transforming growth factor beta (TGF β), IL-6 (251) or/and IL-21 polarises helper T cells into Th17 cells (252). Studies have reported increased Th17 cell frequencies (253-256) and correlation with disease activity or pathological severity (253-260) as well as increased IL-17 serum concentrations (256-259, 261) in SLE patients and mice models of SLE (258, 262). These reports indicate the equivocal nature of the current body of work on T cells and SLE.

In this study a comprehensive phenotypic analysis of myeloid dendritic cells and pDCs, $\gamma\delta$ T cells, Tregs and Th17 cells was performed in a cohort of West Australian lupus patients and controls. We show that IL17 and Th17 cells are not driving inflammation, in contrast, mDC2s and $\gamma\delta$ T cells are involved in the inflammatory response in these patients.

3.3 Results

3.3.1 The proportions of myeloid dendritic cells 1 & 2 are increased in healthy controls compared to SLE patients

Percentages of peripheral blood mononuclear cells (PBMCs) positive for mDC1, mDC2 and pDC, and percentages of lymphocytes positive for gamma delta ($\gamma\delta$), Tregs and Th17 are shown in Table 3.1.

Phenotyping of cells subsets was performed using specific cell surface markers with multi-coloured fluorochrome panels (see methodology p36) that enabled clear discrimination between cell subsets. The proportions of dendritic cells showed an overall decrease in SLE cohort, especially for the mDC1 and mDC2 subtypes ($p < 0.0001$ and $p = 0.0121$ respectively) whereas the proportions of pDCs remained similar (NS) (see Figure 3.1). It was noted that the proportion of mDCs in the SLE patients group was reversed, with a small increase in mDC2 cells compared to mDC1 ($p < 0.0287$) (see Table 3.1). Regulatory T cells proportions were similar in both groups and we observed a small increase in the proportion of Th17 cells in healthy controls

($p = 0.0270$). In contrast, the $\gamma\delta$ T cells subpopulation showed an increased proportion in the SLE group ($p = 0.0767$) (see Figure 3.2).

3.3.2 Intracellular IL17 synthesis by $\gamma\delta$ T cells is increased in SLE patients

Intracellular IL-17 synthesis by Tregs, $\gamma\delta$ T cells and Th17 cells was investigated. We found that the relative amount of intracellular IL-17 was significantly higher in $\gamma\delta$ T cells from SLE patients compared to healthy controls ($p = 0.0010$). There was no meaningful difference in the relative amount of intracellular IL17 synthesised by Tregs and Th17 between the groups (see Table 3.2 & Figure 3.3).

The relative amount of intracellular TGF β was higher in Tregs from SLE patients compared to healthy controls ($p = 0.0019$), but no significant differences between the cohorts in the relative amounts of TGF β synthesised by $\gamma\delta$ T and Th17 cells were seen (see Table 3.2 & Figure 3.4).

3.3.3 Elevated serum pro-inflammatory cytokines in SLE patients

The concentrations of 13 key cytokines were investigated in patients' and controls' serum. Concentrations (+/- 95% confidence intervals (CI)) are shown in Table 3.3. Interleukin 10 (IL-10) appeared elevated in controls, although this difference did not reach statistical significance between the groups (Table 3.3). Briefly, most cytokines measured appeared to be elevated in patients compared to healthy controls with IFN γ TNF α , MCP-1, IL-6, IL-8 and IL-18 attaining significant differences for which the p value is less than 0.05 ($p = 0.0193, 0.0317, 0.0002, 0.0190, 0.0350$ and <0.0001 respectively).

3.3.4 Associations between IL17 and $\gamma\delta$ T cell

Correlation coefficients were estimated for intracellular IL17 synthesis by T cells, DC subsets and several other serological analytes usually measured in SLE. These are shown Table 3.4. In SLE patients, a positive association was observed between the $\gamma\delta$ T cell and mDC2 cell subsets ($p = 0.0408$). Intracellular IL17 concentrations by $\gamma\delta$

T cell were significantly correlated with the $\gamma\delta$ T cell subset ($p < 0.0001$) and mDC2 cells ($p < 0.0001$). The correlation matrix also revealed no associations between IL17A inflammatory cytokine levels and/or intracellular IL17 cytokine levels and Th17 cell subsets in SLE patients. There was a slight correlation between the proportions of intracellular IL17 synthesised by Th17 cells and the concentration in serum of C1q; C3, and DNASE I ($p = 0.0142$; 0.0322 ; 0.0377 , respectively), whereas in the control cohort the concentrations of the inflammatory cytokine IL17A was correlated with $\gamma\delta$ T cell ($p = 0.0162$) (see Table 3.4 & Table 3.5).

3.4 Discussion

The cytokine milieu is a key determinant of the type of immune response elicited, with pro-inflammatory cytokines contributing to the inflammation characteristic of SLE, whilst anti-inflammatory cytokines and chemokines (such as IL10 and TGF β) mediate immune tolerance (of which there is a breakdown in SLE). All cell types can produce TGF β (263), including T cells (264), of which Tregs also synthesise IL10 (265-267). Konkel and colleagues (268) have shown that TGF β signals are crucial for Tregs to regulate tissue-specific immune responses and suppress T helper cell subsets. It was noted that our SLE patients had higher relative proportions than controls of TGF β synthesis by Tregs and $\gamma\delta$ but not by Th17 T cells. Moreover, SLE patients have significantly higher levels of several circulating pro-inflammatory cytokines including IFN γ , TNF α , MCP-1, IL-6, IL-8 and IL-18, implying chronic inflammation. Tregs should exert a regulatory function to maintain tolerance and regulate inflammatory responses (268). However, decreased proportions of peripheral blood Tregs and IL-10 serum concentrations in the SLE patient group relative to healthy controls, together with the lack of TGF β signals from other T cell subsets, implies that in the SLE cohort these functions might be impaired. A number of studies have shown that the regulatory function of Tregs in SLE patients is diminished (269) and in the presence of IL-1 β , (270), IL-23, TNF α , and IL-6 Tregs have been shown to synthesise IL-17 (271, 272) and possess the capability to become Th17 cells (273) that express Foxp3 and the Th17 RAR related orphan receptor C (RORc) transcription factors, hence acquiring an inflammatory phenotype (274). Since Th17 cells in our SLE cohort

are not elevated in comparison with the control cohort, and do not appear to synthesise IL17, the data suggest that Tregs could adopt an inflammatory phenotype; although it seems unlikely these cells would differentiate into mature Th17 cells despite the recognised plasticity between Tregs and Th17 cells (274). Thus, another pathway may also be functional in this cohort of treated SLE patients, i.e. the Th1/Th17 network or one involving $\gamma\delta$ T cells.

In healthy individuals, dendritic cells participate in the removal of apoptotic cells and maintenance of self-tolerance (reviewed by (275)). However, Rosen et al (276) have shown that impaired apoptosis in SLE patients and the presence of apoptotic blebs from cell debris, can generate autoantigens that activate mDCs with consequential loss of tolerance (277). Moreover, defective clearance of apoptotic debris drives autoantibody production and inflammatory responses (278), leading to further DC maturation. Autoantibodies bound to apoptotic cells augment secretion of pro-inflammatory cytokines such as IL-1 β and TNF- α (279). In agreement with other studies (280), we show that the frequency of peripheral blood mDCs is significantly lower in SLE patients than healthy individuals. The proportion of mDC2 cells was elevated compared to mDC1 cells in SLE patients. This reflects a reversal of the mDC1 to mDC2 ratio seen in healthy humans in which mDC1s are the major myeloid DC subset in blood (217). Poulin and others demonstrated a superior uptake capability by mDC2 to present antigens to CD8⁺ T cells (218); upon ingestion of apoptotic blebs, mDCs mature (215) and mDC2 cross-present antigens from effete and necrotic cells, (218, 281, 282). Accordingly, in this study a rapid sequestration of DCs out of the circulation and into tissues, and skewing towards mDC2-mediated uptake of dead and dying cells in tissues promoting Th1 responses could be implied, rather than the mDC1 specialised CD4⁺ T cell activation toward Th17 cells (283). A number of studies have also shown reciprocal regulation of $\gamma\delta$ T cells and DCs (284-286), and in SLE patients, we observed a positive correlation between the two subsets. DCs foster IL-17, IL-21 and IL-22 production by $\gamma\delta$ T cells (284, 286) and IL-1 β and IL-23 influence $\gamma\delta$ T cell activation independent of the T cell receptor (287); both of these cytokines were elevated in SLE compared to healthy controls. The relative frequency of $\gamma\delta$ T cells was increased in SLE patients relative to controls, and was associated with $\gamma\delta$ T

cell intracellular IL17 concentrations. Moreover, we observed increased concentrations of IFN γ and IL-17A in serum, as well as increased intracellular TGF β and IL-17 synthesis by $\gamma\delta$ T cells. Interestingly, $\gamma\delta$ T cells can function as antigen presenting cells and induce apoptosis in cells at sites of inflammation (285).

These data suggest the existence of a mDC2/ $\gamma\delta$ T cell axis that promotes aberrant inflammatory reactions and autoimmunity in SLE patients *in vivo*, mediated by a possible dual function of $\gamma\delta$ T cells in presenting antigens released from effete cells and mediating IL-17-driven inflammation. A feature of this study was the use of fresh blood samples that had not been subjected to laboratory manipulations. Treatment regimens in this cohort might influence T cell outcomes. Steroids and immunosuppressive drugs increase levels of Foxp3⁺ T cells and serum IL-10 concentrations (288) in SLE but not healthy cohorts (289), and in this environment, Th17 might be subdued, while the environment might be conducive to $\gamma\delta$ T cells.

We conclude that Th17 and IL17 mediated inflammation does not appear to be the cause of SLE pathology in this SLE cohort. It is a possibility that increased serum levels of pro-inflammatory cytokines and chemokines and increased proportion of IL-17⁺ and TGF β ⁺ $\gamma\delta$ T cells might contribute to a persistent chronic inflammatory state in patients with treated SLE. Certainly, the treatment regimens in this group of patients seems to suppress this pathway of inflammation whilst promoting a mDC2/ $\gamma\delta$ T cell axis. This axis has not been investigated extensively and further work is required to determine its relevance and role in the pathogenesis of SLE.

3.5 Tables and figures

3.5.1 Tables

Table 3.1: Cell subsets frequencies in SLE patients and controls

		mDC1	mDC2	pDC	$\gamma\delta$	Tregs	Th17
SLE	N	55	55	55	52	55	55
	Mean (%) & 95% CI	0.1096 (0.0853–0.1339)	0.1774 (0.1303–0.2244)	0.3650 (0.3202–0.4099)	0.2850 (0.1937–0.3763)	0.1863 (0.1433–0.2293)	0.0344 (0.0116–0.0573)
HC	N	33	33	33	33	33	33
	Mean (%) & 95% CI	0.2471 (0.1796–0.3145)	0.2320 (0.1799–0.2840)	0.3422 (0.2948–0.3897)	0.1406 (0.0985–0.1828)	0.2332 (0.1765–0.2900)	0.0629 (0.0276–0.0982)
<i>P</i> value		< 0.0001	0.0121	0.4619	0.0767	0.1417	0.0227

Results show percentages of positive cells for each subtype. Dendritic cells are percentages of peripheral blood gated mononuclear cells positive whilst T cells are percentages of gated lymphocytes positive. The table shows the number of values (N), mean concentrations and 95% confidence intervals (Mean; 95% CI). mDC1 = myeloid dendritic cells type 1; mDC2 = myeloid dendritic cells type 2; pDC = plasmacytoid dendritic cells; $\gamma\delta$ = gamma delta T cells; Tregs = regulatory T cells; Th17 = T helper cells 17. *P*-values from Mann Whitney rank test.

Table 3.2: Interleukin 17 & TGF β synthesis in SLE patients and controls

		IL17 by Tregs	IL17 by $\gamma\delta$	IL17 by Th17	TGF β by Tregs	TGF β by $\gamma\delta$	TGF β by Th17
SLE	N	55	53	55	55	53	55
	Mean (%) & 95% CI	0.03375 (0.0108–0.0573)	0.1368 (0.0722–0.2014)	0.0100 (0.0007–0.0192)	0.0621 (0.0418–0.0823)	0.1603 (0.0904–0.2301)	0.0158 (0.0034–0.0282)
HC	N	33	33	33	33	33	33
	Mean (%) & 95% CI	0.0174 (0.0023–0.0323)	0.0310 (0.0145–0.0474)	0.0078 (-0.0004–0.0158)	0.0315 (0.0010–0.0531)	0.0572 (0.0359–0.0786)	0.0182 (-0.0017–0.0382)
<i>P</i> value		0.3345	0.0010	0.7457	0.0019	0.1451	0.1386

Results show percentages of IL17 positive cells. The table shows the number of values (N), mean concentrations and 95% confidence intervals (Mean; 95% CI). $\gamma\delta$ = gamma delta T cells; Tregs = regulatory T cells; Th17 = T helper cells 17; TGF β = transforming growth factor beta. *P*-values from Mann Whitney rank test.

Table 3.3: Cytokine bead array concentrations of analytes in SLE patients and controls

	Patients		Healthy Controls	
	n	Mean concentrations & 95% CI	n	Mean concentrations & 95% CI
IL-1 β	56	305.6 (122.7 – 488.5)	33	183 (0 – 393.1)
IFN α	56	697.6 (327.8 – 1067)	33	342.7 (28.9 – 656.6)
IFN γ	56	2483 (1856 – 3110)	33	1477 (721.1 – 2233)
TNF α	56	3114 (1101 – 5127)	33	1108 (0 – 2319)
MCP-1	56	1808 (1463 – 2154)	33	854 (576.8 – 1131)
IL-6	56	408.2 (138.4 – 678.1)	33	198.2 (0 – 415.2)
IL-8	56	503.9 (118 – 889.8)	33	145.2 (1.158 – 289.3)
IL-10	56	263.5 (79.69 – 447.3)	33	309.2 (0 – 769.3)
IL-12p70	56	192.2 (48.63 – 335.8)	33	136.3 (0 – 302.6)
IL-17A	56	597.7 (119.5 – 1076)	33	160.6 (37.81 – 283.4)
IL-18	56	1071 (801.1 – 1340)	33	378.5 (193.6 – 563.3)
IL-23	56	1136 (20.26 – 2251)	33	196.1 (16.53 – 375.6)
IL-33	56	7414 (2595 – 11432)	33	4494 (0 – 9222)

Results show concentrations (pg/mL) for each analyte. The table shows mean and 95% confidence intervals. IL-1 β : interleukin 1 beta, IFN α : interferon alpha, IFN γ : interferon gamma, TNF α : tumour necrosis factor alpha, MCP-1: monocyte chemoattractant 1 (CCL2), IL-6: interleukin 6, IL-8: interleukin 8, IL-10: interleukin 10, IL-12p70: interleukin 12p70, IL-17A: interleukin 17A, IL-18: interleukin 18, IL-23: interleukin 23, and IL-33: interleukin 33.

Table 3.4: Correlation matrix of cellular and serological parameters for SLE patients

	$\gamma\delta$	Th17	IL17 Tregs	IL17 $\gamma\delta$	IL17 Th17
C1q	0.1342	0.4525 ($p = 0.0005$)	-0.0757	0.1894	0.3289 ($p = 0.0142$)
C3	0.1134	0.0772	0.1233	0.3495 ($p = 0.0103$)	0.2893 ($p = 0.0322$)
α C1q	0.0716	-0.1487	-0.2477	-0.0913	-0.3596 ($p = 0.0070$)
DNASE I	-0.0287	0.2186	-0.0953	0.0114	0.2869 ($p = 0.0337$)
mDC2	0.2847 ($p = 0.0408$)	-0.1479	0.0901	0.5313 ($p < 0.0001$)	-0.0776
Tregs	0.2804	0.3072	0.4442 ($p = 0.0007$)	0.1320	0.1349
$\gamma\delta$		0.4064	0.2420	0.5327 ($p < 0.0001$)	0.1412

The Table shows the Pearson correlation coefficients, and when applicable and of interest, the p value.

Table 3.5: Correlation matrix of cellular and serological parameters for healthy controls

	mDC1	pDCs	$\gamma\delta$
IL17 $\gamma\delta$	0.3802 ($p = 0.0290$)	0.0417	0.1100
MCP-1	-0.2350	0.4276 ($p = 0.0131$)	-0.0473
IL-8	-0.0575	0.4766 ($p = 0.0050$)	-0.0432
IL-17A	-0.1790	-0.0113	0.4154 ($p = 0.0162$)
IL-23	-0.0209	0.4638 ($p = 0.0066$)	-0.0553

The Table shows the Pearson correlation coefficients, and when applicable and of interest, the p value.

3.5.2 Figures

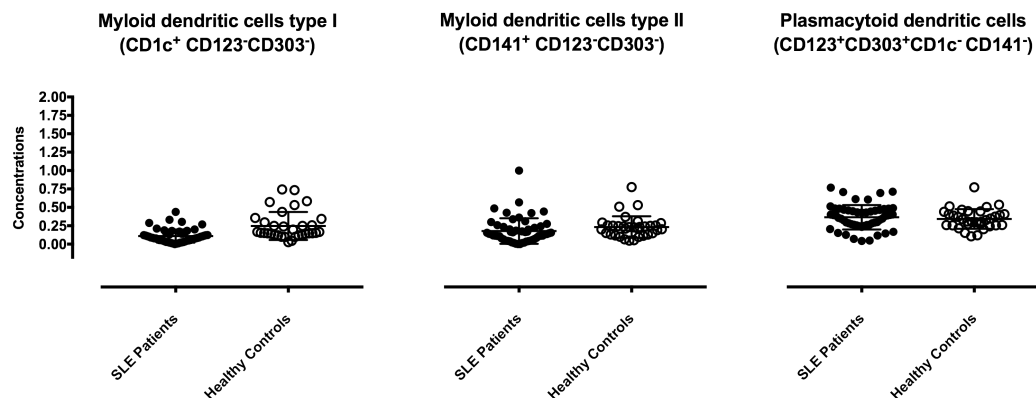


Figure 3.1: Dendritic cell profiles in SLE patients and healthy controls.

Cell subtypes are based on specific cell markers as indicated on the graph. The figure shows the cell population expressed as percentages of peripheral blood mononuclear cells (X-axis) for SLE patients (dark circles) and the control cohort (light circles). Each graph shows the mean and standard deviation for each group; mDC1 $p < 0.0001$; mDC2 $p = 0.0121$; pDCs NS ($p = 0.4617$). (Mann Whitney rank test). Mean concentrations are shown in Table 3.1.

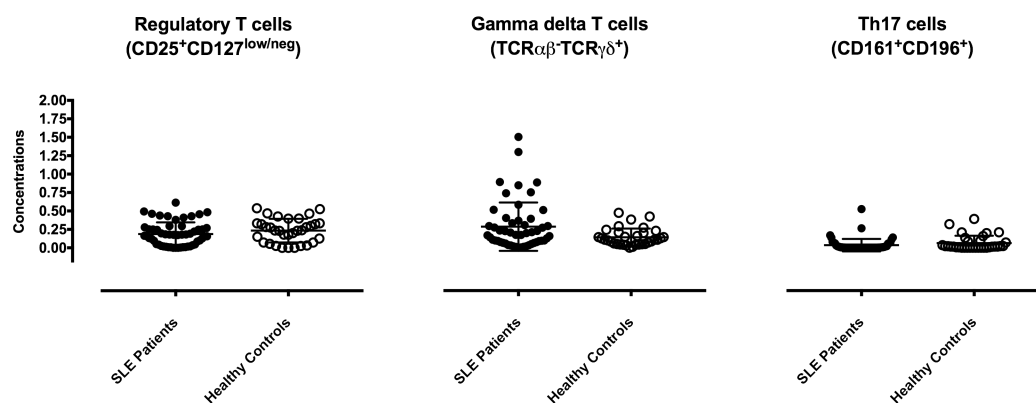


Figure 3.2: T cell profiles in SLE patients and healthy controls.

Cell subtypes are based on specific cell markers as indicated on the graph. The figure shows cell concentrations expressed as percentages of lymphocytes (X-axis) for SLE patients (dark circles) and the control cohort (light circles). Each graph shows the mean and standard deviation for each group; Tregs NS ($p = 0.1417$); Gamma Delta NS ($p = 0.0767$); Th17 $p = 0.0270$. (Mann Whitney rank test). Mean concentrations are shown in Table 3.1.

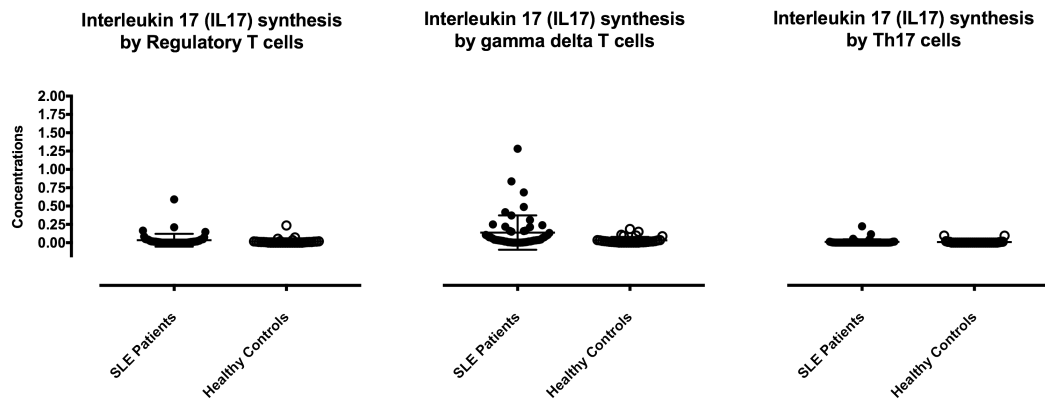


Figure 3.3: Interleukin 17 synthesis in SLE patients and healthy controls

The figure shows cell populations expressed as percentages of IL17 positive cells (X-axis) for SLE patients (dark circles) and the control cohort (light circles). Each graph shows the mean and standard deviation for each group; IL17 synthesised by Tregs NS ($p = 0.3345$); IL17 synthesised by Gamma Delta $p = 0.0010$; IL17 by synthesised Th17 NS ($p = 0.0767$) (Mann Whitney rank test). Mean concentrations are shown in Table 3.2. $\gamma\delta$ = gamma delta T cells; Tregs = regulatory T cells; Th17 = T helper cells 17.

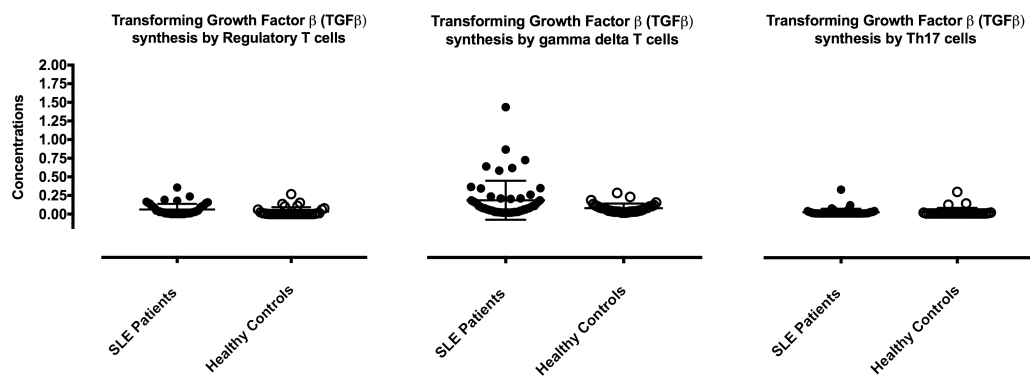


Figure 3.4: Transforming Growth Factor β (TGF β) synthesis in SLE patients and healthy controls

The figure shows cell concentrations expressed as percentages of TGF β positive cells (X-axis) for SLE patients (dark circles) and the control cohort (light circles). Each graph shows the mean and standard deviation for each group; TGF β synthesised by Tregs $p = 0.0019$; TGF β synthesised by Gamma Delta NS ($p = 0.1451$); TGF β synthesised by Th17 NS ($p = 0.1386$) (Mann Whitney rank test). Mean concentrations are shown in Table 3.2. $\gamma\delta$ = gamma delta T cells; Tregs = regulatory T cells; Th17 = T helper cells 17.

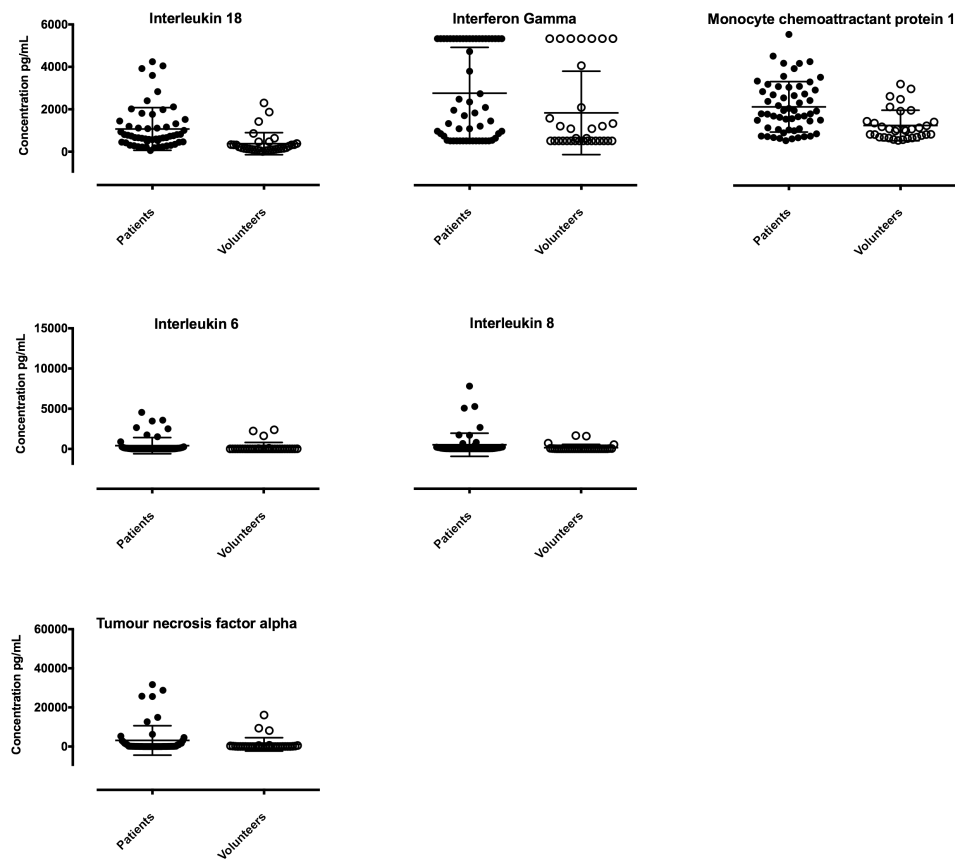


Figure 3.5: Cytokine bead array assay 13-plex.

The figure shows cytokine concentrations (pg/mL) (X-axis) for SLE patients (dark circles) and the control cohort (light circles). Each graph shows the mean and standard deviation for each group; interleukin 18 $p = 0.0350$; interferon gamma $p = 0.0193$; monocyte chemoattractant protein 1 $p = 0.0002$; interleukin 6 $p = 0.0190$; interleukin 8 $p = 0.0350$; tumour necrosis factor alpha $p = 0.0317$ (Mann Whitney rank test). Mean concentrations are shown in Table 3.3.

Chapter 4. Anti-C1q Antibodies Concentrations by ELISA in Systemic Lupus Erythematosus

The content of this chapter has been published in *Lupus: Open Access* (2017). Permissions for this article can be found in appendix B.1.2 and published article in B.2.2.

Margery-Muir A A. Wetherall J D. Groth D M. and Bundel C. *Anti-C1q Antibodies Concentrations by Elisa in Systemic Lupus Erythematosus*. *Lupus: Open Access*. 2017. **2**(1): p. 124.

4.1 Abstract

Systemic lupus erythematosus (SLE) is an inflammatory disorder in which autoantibodies contribute to impaired apoptosis and clearance of cell debris. Anti dsDNA and anti C1q antibodies have been implicated, as well as complement protein C1q itself. IgG autoantibodies reacting with the collagen-like region of C1q protein (henceforth α C1q ab) were quantitated in serum of 56 patients diagnosed with SLE and undergoing treatment for variable periods, together with 33 age/sex-matched controls. Analysis of the results showed optimal sensitivity and specificity of 57% and 91% respectively at a cut-off concentration for positivity of 20 U/ml. The assay is a potentially useful confirmatory test for SLE, but is not suitable as a screening test for SLE with the probability of a positive test and SLE in an individual within a random population of only $\leq 1\%$. α C1q ab concentrations were detectable in all samples tested with concentrations manifesting no correlation with age and serum C1q levels in SLE patients and a negative correlation with age in controls. The α C1q ab detected by this assay do not react therefore with native C1q. In SLE patients, α C1q ab concentrations correlated with the concentrations of dsDNA antibodies, ($p = 0.0001$) and C-reactive protein and inversely with complement component C4 ([C4]) concentrations ($p = 0.041$). α C1q ab concentrations were not associated with individual therapeutic regimens, but were higher in those patients receiving a combination of three drug therapies and with the presence of renal disease. The diagnostic relevance of this complex autoantibody will require further definition of its antigenic specificities.

4.2 Introduction

There is good evidence that impaired apoptosis and clearance of cell debris induces autoimmune responses associated with aberrant inflammation (290). Systemic lupus erythematosus (SLE) is an important systemic autoimmune disease characterised by the presence of autoantibodies that bind double stranded DNA (henceforth dsDNA ab) and often decreased levels of serum complement components C3 and C4 (16). Complement component C1q is one of three proteins comprising the first component

(C1) of the classical pathway of complement activation. C1q molecules can activate the complement system by recognizing different structures on microorganisms and apoptotic bodies, or indirectly by means of binding to immunoglobulins and classical acute phase proteins such as C-reactive protein, (CRP) resulting in clearance of immune complexes and apoptotic material (16, 291). The avidity of binding of C1q to the Fc domain of IgG antibodies is augmented by the presence of hexameric IgG/antigen complexes on cell surfaces or polyvalent antigens (292). A consequence of high affinity binding of C1q to Fc regions is the formation of a neo-antigen on the collagen-like tails of the C1q molecule subunits (293). Production of auto-antibodies reacting with this neo-antigen has been observed following C1q binding with immune complexes or nonspecific binding to chromatin (294). The role of these autoantibodies in the pathogenesis of specific disease, for example SLE, is yet to be clearly defined (16). Uwatoko and colleagues (295) devised an assay capable of quantifying anti-C1q antibodies (α C1q ab) by their interaction with C1q bound to a plastic surface in the presence of a high salt concentration (1M NaCl) to preclude any interaction with soluble C1q present in the test sample, usually serum.

In the last decade, the relationship between α C1q ab and clinical profiles in SLE patients, as well as relationships with other laboratory parameters has been investigated. It has been reported by several groups (294, 296-298) that the levels of α C1q ab titers are usually significantly higher in SLE patients than healthy controls or non-SLE autoimmune patients. The concentrations of α C1q ab correlate with disease activity and/or severity (294, 296-307) as well as with other laboratory parameters including complement protein C3 and/or C4 concentrations, dsDNA ab, proteinuria and the presence of immune complexes (294, 296, 299, 300, 302-307). Whether serum concentration of α C1q ab is a useful tool to predict up-coming flares in patients with nephritis remains to be confirmed. A strong association between α C1q ab and active renal disease has been demonstrated (306). Only nine studies have longitudinal data permitting this specific assessment (including (298, 302)), although other reports have supported its potential for this use (298, 300, 305). The present study measured the levels of α C1q ab in a cohort of diagnosed and treated Western Australian SLE patients using a commercial ELISA kit.

4.3 Results

4.3.1 Quantitation of anti-C1q antibody in treated SLE patients and controls

Multiple assays (including repeated samples) were performed in accordance with the manufacturers specifications using a range of international calibrator concentration standards (5, 25, 100 and 400 U/ml) provided with the kit. Two quality control samples provided by the manufacturer were included in each assay. These comprised a negative control with expected values between 4.0-7.9 U/mL and a positive control sample between 118-233 U/mL. The positive control was always within the expected concentration range. The negative control sample was marginally higher in our analysis (average of 9.9U/mL) than that stipulated by Buhlmann Ltd, but still lower than the 20 U/mL cut-off point for positivity used in this analysis (see Figure 4.1).

The non-Gaussian distributions of the concentration of the α C1q ab in patients and controls are show in Figure 4.2. The optimal specificity and sensitivity of the assay for inclusion or exclusion of SLE were estimated from the proportions of true positive and true negative results as a function of variable “cut-off” concentrations of α C1q ab. From these data, a receiver-operator characteristic curve (ROC: plot of sensitivity (%) versus 1-specificity (%), see Figure 4.3) was generated with an area under the curve of 0.78 (0.68 -0.88, 95% CI $p = 0.0001$). From this graph, the 20 U/mL cut-off concentration provided a likelihood ratio of 6.3 (ie. probability of a positive test in patients with SLE/probability of a positive test in persons without SLE). This compares with the likelihood ratio of 5.0 for the 15 U/mL cut-off value recommended by the manufacturer, and often quoted by other groups using the same international control samples (294). Henceforth the 20 U/mL discrimination value has been used throughout this analysis. The sensitivity and specificity of the assay for the diagnosis of SLE were 57% and 91% respectively (see Table A 3; Table A 4; Table A 5; Table A 6). Assuming an average prevalence of SLE of $\approx 0.1\%$ in the WA population (308), the estimated predictive value of a positive and negative α C1q ab test for SLE was 91% and 56% respectively. Bayesian analysis results in the probability of a positive test

and SLE in an individual within a random population of the order of 1-2%. There was a higher percentage of positive α C1q ab concentrations in SLE patients than in controls (57% v 9% respectively, $p = 0.0001$; Mann Whitney rank test), and it was noted that all patients and control samples tested manifested some level of α C1q ab.

4.3.2 Anti-C1q antibody concentrations correlate in SLE patients with dsDNA ab concentrations and inversely with complement C4 concentrations.

Correlation coefficients were estimated for α C1q ab concentrations and those of several other serological analytes usually measured in SLE testing. These are shown in Table 4.2. Significant correlations were observed between α C1q ab and dsDNA ab ($p = 0.0001$); an inverse correlation between α C1q ab concentrations and complement component C4 concentrations was noted ($p = 0.041$). The correlation matrix also revealed associations in SLE patients between C-Reactive Protein (CRP) and both dsDNA ab ($p = 0.005$) and C1q complement protein levels ($p = 0.024$). However, C1q protein levels in SLE patients were not significantly correlated with anti-dsDNA antibodies. There was a weak negative correlation (not statistically significant), between the age at time of testing of individual SLE patients and the concentration in serum of α C1q ab, whereas in the control cohort the concentration of α C1q ab was negatively correlated with age at time of collection ($p = 0.001$).

4.3.3 Anti-C1q ab concentrations are associated with treatments and renal co-morbidities in patients

Heatmaps were used to show any patterns in the patient group linking concentrations of α C1q ab and therapeutic treatments, as well as co-morbidities. Figure 4.4A shows the association of the α C1q ab with the drug treatments offered to SLE patients over the course of their disease. It was observed that patients who received all three treatments of steroids, anti-malarial and immune-suppressants (7th lane) exhibited higher levels of α C1q abs. Figure 4.4B shows associations of α C1q abs with the co-morbidities of the patients. The first lane shows an association of higher concentrations of α C1q abs in patients with diagnosed renal disease. In

contrast, patients with hepatic involvement (3rd lane) exhibit lower levels of α C1q abs.

4.4 Discussion

C1q molecules can activate the complement system by recognizing different structures on microorganisms and apoptotic bodies, or indirectly by means of bound immunoglobulins and classical acute phase proteins such as C-reactive protein, (CRP) (291). Following these processes the generation of a neo antigen on the α C1q ab concentrations were assayed in the serum of a cohort of SLE patients undergoing clinical management for varying durations, and a panel of 33 healthy controls using a commercially available ELISA (Buhlmann Laboratories). The merits of the Buhlmann assay for α C1q ab are its commercial availability (including concentration standards), relative ease of use and reproducibility in a diagnostic laboratory setting. The ROC graph analysis of the test result data showed that 20 U/mL was a more discriminatory cut-off concentration than the value of 15 U/mL recommended by the manufacturer and some other groups (296). The relatively low sensitivity of the assay (57%) restricts the utility of the test for exclusion of SLE, however the high specificity of the assay (91%) provides a useful confirmatory test. Further, the probability of a positive test AND SLE in an individual in a random population is only of the order of 1-2%, thereby ruling out the assay as a screening test for SLE. It is noted that three of the 33 serum samples from the healthy control cohort exhibited high levels of α C1q ab; we cannot explain this anomaly.

This study reports a one point-in-time estimate of α C1q ab concentrations in the serum of a cohort of SLE patients under clinical management for varying durations. Hence, it is not possible in this study to relate α C1q ab levels to events of clinical significance such as flare of renal disease. α C1q ab levels were inversely correlated with serum C3 and C4 concentrations in SLE patients (albeit C3 not significantly) as would be expected if classical pathway complement activation was contributing to the disease process (296), suggesting that in SLE patients, some of these analytes contributed directly to the disease mechanism (309). An important cognate study (310) demonstrated two human monoclonal anti-DNA antibodies (R4A and G11) that

cross-reacted with a globular region (as opposed to the collagen-like region) antigenic determinant in mouse and human C1q proteins. Both antibodies bound both soluble C1q as well as solid phase C1q. Franchin et al., (310) postulated that anti-C1q antibodies could contribute to the development of immune complex mediated glomerulonephritis by direct binding to glomeruli or by removal of soluble C1q. In our study α C1q ab concentrations were not correlated with serum concentrations of C1q in either the patient or control cohorts. This implies that most of the α C1q ab detected in this assay are specific for non-globular antigenic epitopes on C1q. The significant correlation of α C1q ab concentration with anti-dsDNA antibodies is consistent with the cross-reactivity reported by Franchin et al., (310), but may also just reflect two separate but related processes. It is also of interest that Vanhecke et al., (311) described α C1q ab reacting with peptide epitopes on the A and B collagen-like tails of human C1q that were not present on the C chain. These epitopes were only present on bound C1q and not on soluble C1q and mapped to different regions of the collagen-like region of C1q. This raises the possibility that the α C1q ab detected in this study may include a proportion of antibodies directed against the peptide epitopes described by Vanhecke (311). It is clear the specificities of the α C1q ab observed in the serum of SLE patients need to be characterised in more detail, especially if different antibody specificities reflect distinct attributes of SLE pathology, for example presence of nephritis.

α C1q ab occur in the serum of individuals with a variety of non-SLE autoimmune disorders and infectious diseases, as well as in a proportion of healthy individuals (reviewed by 293). It is clear the presence of α C1q ab as detected by the ELISA assay used herein is not pathognomonic for SLE. The enigmatic role of C1q in SLE is emphasized further by the recent review (by international survey) of C1q deficiency in humans (76) in which the authors reported a high prevalence (\approx 80%) of SLE, together with decreased survival times and increased susceptibility to infections.

It seems there are at least two distinct mechanisms in the predisposition to SLE explained by C1q. SLE occurs with high frequency in C1q protein deficient persons (76, 312) and SLE can also occur in the presence of normal C1q protein levels and relatively high concentrations of α C1q ab that contribute to inflammatory processes

(304). SLE patients who received all three classes of drug therapies tended to have higher levels of α C1q ab. This suggests that patients with a more severe disease phenotype requiring multiple therapeutic treatments to reduce organ damage also produce higher levels of α C1q ab. In contrast, patients with less severe disease, requiring fewer therapeutic agents and manifesting lower levels of α C1q ab. This observation supports, but does not confirm, reports that relate high α C1q ab levels to renal symptoms and/or renal flares (306). There have been many studies over the past two decades of α C1q ab levels in disease, including SLE (305, 306). These, together with this report, suggest that quantitation and standardization of anti-C1q antibodies has a useful role in the classification of subtypes of SLE and as a potential confirmatory diagnostic and monitoring assay.

4.5 Tables and figures

4.5.1 Tables

Table 4.1: Combination of treatment regimens and co-morbidities in SLE patients.

	Steroids (1)	Anti- malarial (2)	Immuno- suppressants (3)	1 + 2	1 + 3	2 + 3	Combination of 1, 2 & 3	Other therapeutics
Renal symptoms (N=11)	8 (72.7%)	7 (63.6%)	8 (72.7%)	1 (9.1%)	3 (27.3%)	0 (0%)	4 (36.4%)	8 (72.7%)
Hepatic involvement (N=4)	2 (50%)	4 (100%)	2 (50%)	1 (25%)	0 (0%)	1 (25%)	1 (25%)	3 (75%)
Anti-phospholipid (N=17)	13 (76.5%)	9 (52.9%)	7 (41.2%)	3 (17.6%)	3 (17.6%)	0 (0%)	4 (23.5%)	16 (94.1%)
Raynaud's phenomenon (N=4)	2 (50%)	2 (50%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	4 (100%)
Sjögren's disease (N=4)	0 (0%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (75%)
Cutaneous/discoid symptoms (N=12)	5 (41.7%)	6 (50%)	7 (58.3%)	1 (8.3%)	0 (0%)	0 (0%)	3 (25%)	9 (75%)
Neuronal symptoms (N=1)	1 (100%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
T1DM (N=3)	3 (100%)	2 (66.7%)	2 (66.7%)	1 (33.3%)	1 (33.3%)	0 (0%)	1 (33.3%)	3 (100%)
Other symptoms (N=13)	9 (69.2%)	11 (84.6%)	7 (53.8%)	2 (15.4%)	2 (15.4%)	1 (7.7%)	4 (30.8%)	13 (100%)

The table indicates the number and percentage of patients in each category of lupus manifestation under specific types of treatment (Patients may fit into multiple lupus manifestations as well as treatments under review with other type of medications).

Table 4.2: Correlation matrix between α C1q ab and other serological parameters and age.

	Age	[C4]	(C1q)	(C3)	(CRP)	(Ds-DNA ab)	(DNASE-I)	(Anti-C1q ab)
Age		-0.073	0.070	0.063	0.014	-0.275 ($P=0.042$)	0.034	-0.146
[C4]	-0.073		0.085	0.653 ($p \leq 0.0001$)	0.203	-0.087	-0.045	-0.274 ($P=0.041$)
(C1q)	0.070	0.085		0.248	0.325 ($P=0.024$)	0.241	0.263 ($P=0.050$)	0.088
(C3)	0.006	0.116	-0.020		0.239	-0.118	0.017	-0.131
(CRP)						0.406 ($P=0.005$)	-0.063	0.136
(Ds-DNA ab)							0.059	0.502 ≤ 0.0001
(DNASE I)	0.140	-0.141	0.230	0.225				0.038
(Anti-C1q ab)	-0.566 ($P=0.001$)	-0.246	-0.024	0.057			-0.368 ($P=0.035$)	

The values above the diagonal are the Spearman (r) correlation values and when significant the significance levels in brackets ($p \leq 0.05$). The same comparison when available, are shown for the control population below the diagonal line.

4.5.2 Figures

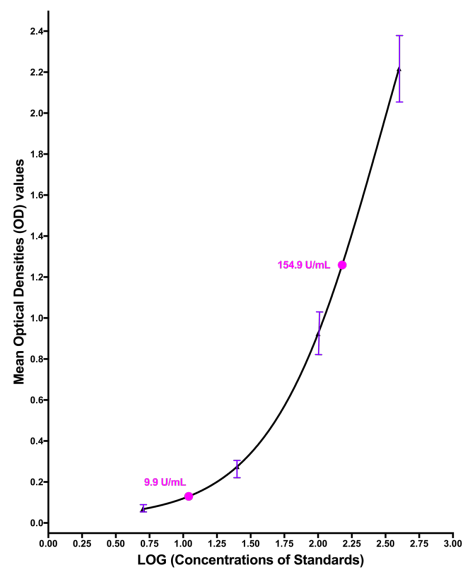


Figure 4.1: A four-parameter logistic standard curve for the α C1q ab kit.

Curve of the assay (Buhlmann Laboratories) over the range of 5, 25, 100 and 400 U/ml (mean \pm standard deviation of optical density values).

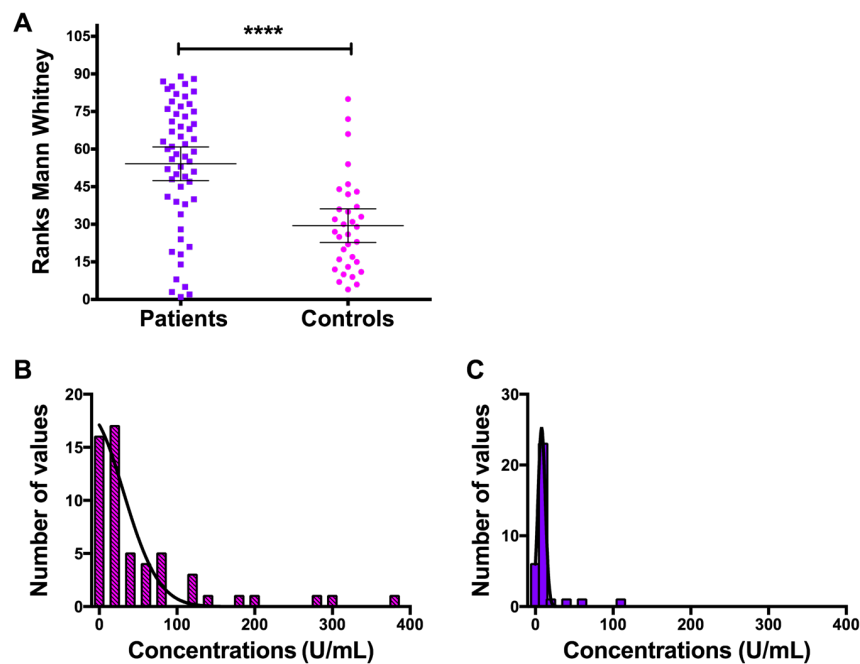


Figure 4.2: α C1q ab distribution and ranking test.

Figure 3.3A: Ranking of anti-C1q concentrations and means of ranks of α C1q ab \pm 95% CI (Mann Whitney rank test analysis $p < 0.0001$). Figure 3B & 3C: Frequency distribution of the α C1q ab for SLE patients and controls respectively (mean concentrations - α C1q ab SLE patients: 54.70 U/mL; controls: 12.84 U/mL).

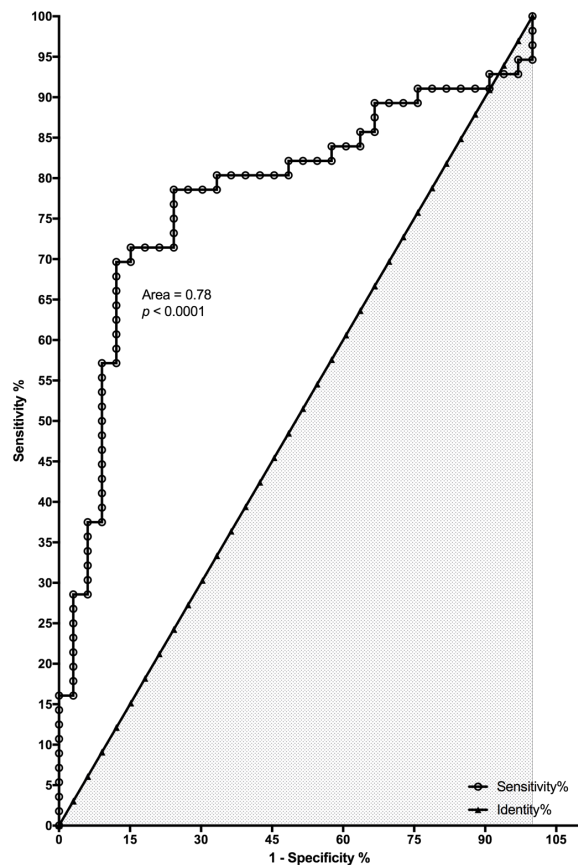


Figure 4.3: Receiver operating characteristics (ROC) curve for quantitation of α C1q ab.

The area above the diagonal line corresponds to 78% of the total area indicating good discrimination between disease and non-disease at the 20 U/ml cut-off level ($p < 0.0001$).

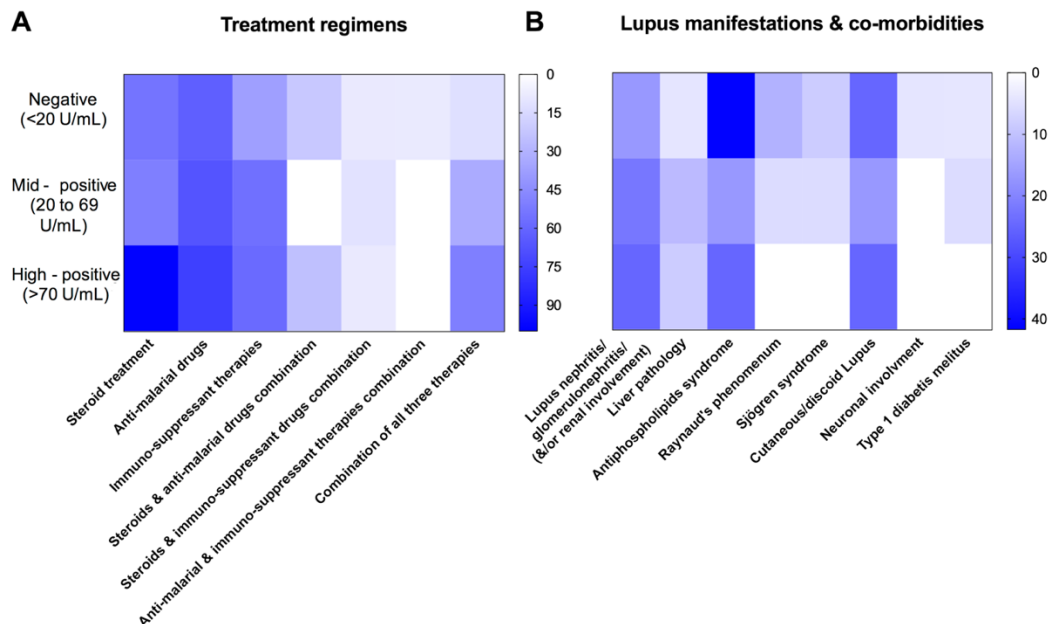


Figure 4.4: Heatmaps of treatment regimens and SLE-comorbidities associated with α C1q antibody concentrations.

The figure shows associations between α -C1q antibody concentrations (rows) with A. drug therapies and B. co-morbidities (columns). α C1q ab concentrations were classified as negative (<20 U/mL), mid positive (20 to 69 U/mL) and highly positive (> 70 U/mL).

Chapter 5. Insights on the relationship between complement component C4 serum concentrations and C4 gene copy numbers in a Western Australian systemic lupus erythematosus cohort

The content of this chapter has been submitted and is under review with the Journal of Clinical Immunology. Item indicating review process can be found in appendix B, P.150.

Margery-Muir A A. Bundell C. Wetherall J D. Whidborne R. Martinez P. and Groth D. *Insights on the relationship between complement component C4 serum concentrations and C4 gene copy numbers in a Western Australian systemic lupus erythematosus cohort.*

5.1 Abstract

Purpose: The relationship between serum concentration of complement C4 ([C4]) and C4 gene copy number (GCN) variation was investigated in 56 SLE patients and 33 age and sex matched controls in the Western Australian population.

Methods: C4A and C4B genes (C4A & B) GCN were estimated by a TaqMan™ assay and compared with a custom RT-PCR assay that detected C4 genes with and without a ≈6.4kb retroviral element in intron 9 (hereafter HERV-K).

Results: Good agreement between the two methods was observed, although the total count by the HERV-K method showed a GCN dependent bias relative to the C4A & B total GCN. Regression analysis revealed an excellent correlation between C4A & B GCN and [C4] in both SLE and control cohorts ($R^2 = 88\%$ and $R^2 = 99\%$ respectively), however individual variability in serum [C4] precluded estimating GCN for specific individuals. The data reported herein agree with the reference ranges for [C4] reported previously. Serum [C4] was significantly lower in the SLE patients than the controls ($p = 0.006$) but no statistical difference in C4A GCN was found between the groups. The data confirmed previous reports that the C4A genes were preferentially associated with the presence of the HERV-K insertion relative to C4B genes. There was no evidence to show that the presence of the HERV-K insertion in C4 genes influenced [C4].

Conclusion: This study supports the view that low [C4] in SLE patients is due to consumption rather than deficient synthesis related to lower C4A & B GCN.

5.2 Introduction

Complement C4 genes (C4), located within the central region of the major histocompatibility complex (MHC) on the short arm of chromosome 6 (C6p), displays strong linkage disequilibrium with neighbouring loci (313) and contributes to the identity of extended haplotypes that characterize this genomic region (313). C4 proteins are synthesized from the mRNAs of two expressed paralogous genes, designated C4A and C4B (81), resulting in the synthesis of proteins, sharing 99% identity and differing by four isotype-specific amino acids in exon 26 (314). Complement C4 genes are part of a sub-genomic region designated “RCCX” (82, 315, 316). An RCCX module contains loci for Serine/Threonine nuclear protein kinase RP (STK 19, OMIM*604977), complement C4 (C4A &/or C4B, OMIM*120810 & 120820 respectively), steroid 21-hydroxylase CYP21 (CYP-21, OMIM*613815) and the extra cellular matrix protein tenascin X (TNX, OMIM*600985); up to four copies of the module may be present on each copy of C6p, although most individuals have only one per haplotype and hence two copies of each of the C4A and C4B genes (82).

Both loci may also exhibit size polymorphism due to the presence of a \approx 6.4kb HERV-K retroviral insertion in intron nine, resulting in “long” (presence of the retroviral element) and “short” (absence of the retroviral element) C4 gene variants (317, 318). The antisense insertion and lack of promoter activity from the 5’ LTR (both sense and antisense) and the 3’ LTR sense orientation preclude a deleterious effect on the host genes, in this case complement C4 (317). Nevertheless, Yang and colleagues (318) recently reported that the presence of the retroviral element is associated with lower C4 serum concentrations and decreased haemolytic titres of complement activity.

Furthermore, C4A is associated with the presence of a CT insertion in exon 29 (319) that results in a frameshift mutation and a stop codon that effects translation of a non-functional C4 protein (320). Reports suggest that the CT insertion is associated with C4A pseudogenes (320), is present in some C4B genes (320, 321) and predisposes to systemic lupus erythematosus (SLE). Paakkanen and colleagues have shown this CT insertion in more than 70% of the individuals exhibiting C4A deficiency states, and that its prevalence is increased in SLE patients relative to controls (85).

However, the association between the CT insertion and C4A deficiency has not been demonstrated in a recent study of populations from the United Kingdom and Spain (322).

The purpose of this study was to evaluate the levels of complement C4 protein in a cohort of West Australian individuals with SLE, and a sex/ age matched control cohort. The relationship between serum C4 protein concentrations and C4A and C4B gene copy numbers (C4 GCNs) was investigated together with the frequencies of the presence of the HERV-K retroviral element and the exon 29 CT insertion in C4A and C4B genes. In a previous study complement C4 reference ranges for varying C4 GCNs were published to assist clinicians in the interpretation of chronically low serum C4 concentrations in SLE patients (86). The present study extends this work.

5.3 Results

5.3.1 Quantitation of serum C4 concentrations in SLE patients and controls.

Serum samples for the SLE patients and controls were obtained at the time of recruitment. Serum [C4] for the SLE patients show a non-Gaussian distribution with lower concentrations than the controls (Figure 5.1A). Mean [C4] show statistically significant difference between the groups ($p = 0.0060$ – Figure 5.1B).

5.3.2 Measurement of C4 gene copy number (GCN)

C4 GCN's were quantitated in both the SLE and control cohorts using two independent methods as described above. These methods estimate the C4A and C4B GCN and C4 genes with (long) and without (short) the 6.4kb HERV-K retroviral insertion. The results of these measures are summarised in Table 5.1. In the SLE cohort, one individual (1.78%) had C4AQ0 and two (3.57%) had C4BQ0. In the control cohort two individuals (6.06%) had C4AQ0 and none had C4BQ0.

5.3.3 Comparison of C4 GCN by Taqman™ C4A and C4B assay and detection of the HERV-K retroviral insertion by custom Taqman™ assay.

The assays results show good agreement between the two independent methods. A Bland-Altman analysis of the data for the 89 subjects tested showed a small bias of ≈ 0.06 . However, a regression line of the differential gene count versus the means of the two estimates showed a bias that was GCN dependent; low HERV-K counts underestimated total C4A & C4B GCN by ≈ 1 gene whereas high HERV-K counts overestimated the total C4A & C4B GCN by ≈ 1 gene. These results are illustrated in Figure 5.2 and Figure A 7.3 and Figure A 7.4.

5.3.4 Distributions of C4 A and B and C4 long and Short genes in SLE patients and controls

Distributions of C4A, C4B and C4A+C4B with and without the HERV-K retroviral insertion are shown in Figure A 7.5 together with comparable data from Yang et al. (81). The distributions of C4A, C4B and total C4 GCN's and of the long and short variants of these C4 genes were similar to those reported by Yang et al. No significant differences between SLE patients and control cohorts GCN were found in our study.

5.3.5 HERV-K insertion is more frequent in C4A genes than C4B genes

Analysis of the 89 combined SLE patients and controls showed that $\approx 74\%$ of the total C4 genes counted contained the HERV-K insertion. In contrast, the ratio of C4A to C4B genes was ≈ 1 . When the 26 subjects without the HERV-K insertion, or individuals exhibiting only C4 genes with the HERV-K insertion (2) were analysed, the proportion of C4A genes with the HERV-K insertion was 55%. This indicates that the HERV-K insertion is more frequent in C4A genes than C4B genes (1.25:1). Linear regression revealed a positive correlation between C4A genes and the presence of the HERV-K insertion ($r = 0.561$, $p = 0.0001$), while there was no significant correlation between C4A genes and the absence of the HERV-K insertion ($r = 0.038$, $p = 0.726$ NS). In contrast, absence of the HERV-K insertion correlated with the C4B gene ($r = 0.473$, p

≤ 0.0001). There was no significant correlation between C4B genes and the presence of the HERV-K insertion. See Figure 5.3, Figure A 7.6 and Figure A 7.7. These findings are consistent with those of Yang and colleagues who also reported a preferential association of the HERV-K insertion in C4A genes (81).

5.3.6 Serum C4 concentrations as a function of gene copy number variation

The relationship between serum [C4] and C4 GCN in SLE patients and controls was investigated using regression analysis. The intercept of the regression line passes close to the origin with a coefficient of determination of $\approx 40\%$ in healthy controls ($y = 0.0647 \cdot x - 0.003077$). This is consistent with a linear overall relationship between serum [C4] and total C4 GCN with variability between individuals. If the data are aggregated within each GCN, the relationship once again is linear, passing through the origin indicating that (and for which) all variation in serum [C4] is accounted for by GCN ($r^2 = 99\%$; $y = 0.06305 \cdot x + 0.001332$ - Figure 5.4).

An equivalent analysis in 56 SLE patients revealed a similar relationship but with increased individual variation in [C4]. In this analysis, $\approx 12\%$ of the variability in individual serum [C4] was accounted for by total C4 GCN ($y = 0.00452 \cdot x + 0.01846$), while for the aggregated data 88% of the variation in [C4] was related to C4A+B GCN ($y = 0.04711 \cdot x + 0.03347$ - Figure 5.4).

The relationship between serum [C4] and C4 genes with the insertion of the HERV-K retroviral element (long) in SLE patients and controls (aggregated data) was investigated using regression analysis. The intercept of the regression line passes close to the origin with a coefficient of determination of $\approx 25\%$ ($y = 0.04461 \cdot x - 0.01661$ -Figure A 7.8). This is consistent with a linear relationship between serum [C4] and C4 long with variation between. If the data are aggregated within each GCN, the relationship once again is linear, passing through the origin (and for which) all variation in serum [C4] is accounted for by GCN ($r^2 = 98\%$; $y = 0.04524 \cdot x + 0.02175$ - Figure A 7.8). Interestingly, a Mann-Whitney analysis (Figure A 7.8) demonstrated no significant difference in the mean GCN of the C4 genes with the HERV-K element within this subgroup ($p = 0.9595$). However, the analysis demonstrated a statistically

significant difference between the mean [C4] of the two subgroups ($p = 0.0087$) with the SLE patients demonstrating much lower serum concentrations.

5.3.7 The serum C4 concentrations in previously established ranges for two, three, four and five C4 GCN.

We have previously established serum C4 concentration reference ranges for the C4 GCN (86). We compared the data obtained herein with our previously reported data and found that the mean serum C4 concentrations were generally within the ranges previously established in both cohorts with individual variations (see Figure 5.5). In the SLE cohort, 27 (48%) patients have serum C4 concentrations below the internationally used normal range (0.160 – 0.520g/L). Of these 27 individuals, only 16 (28%) have serum [C4] which are still lower than our previously defined ranges (for the C4 GCN determined). One of these patients, with quiescent disease activity has a total of six C4 genes (for which we have no ranges), chronically low serum [C4] of 0.0933g/L together with low serum C3 concentration. However, the antibody profile for anti-C1q, ANA and dsDNA is within the reference range). Therefore, chronically low serum C4 concentrations in this patient must likely be attributed to other factors. Previously defined normal serum C4 mean concentrations and ranges (86) for two, three, four and five total C4 GCN respectively ((0.197; 0.059 – 0.343) (0.256; 0.117 – 0.383) (0.296; 0.159 – 0.439) (0.425; 0.279 – 0.571)).

5.3.8 The CT insertion is not a risk factor in the Western Australian population

All 89 subjects were tested for the presence of the CT insertion in exon 29 of C4 genes, the presence of which precludes C4 protein synthesis (323). Only one of 56 SLE patients (1.8%) and no healthy controls was shown to have this insertion.

5.4 Discussion

Decreased serum concentrations of complement component C4 have long been a valuable aid in the diagnosis and management of SLE (324). The presence of two polymorphic C4 genes, designated C4A and C4B, within the central major

histocompatibility complex has added much interest in the role of these proteins in the pathogenesis of SLE (325). Further, interpreting serum [C4] in the diagnosis and management of SLE may be difficult because low concentrations may reflect consumption or low GCNs and/or the presence of null alleles (324). In this study serum C4 concentrations were significantly lower in 56 confirmed SLE patients than in 33 sex and age matched control persons ($p = 0.006$). There was no evidence that lower serum C4 concentrations in the SLE cohort was due to paucity of C4A and/or C4B GCNs. Only one SLE patient was C4A gene deficient compared with two control individuals; there were two SLE patients and no control individuals deficient in C4B genes. These results preclude C4A deficiency as a risk factor for SLE and support the extensive investigation from Boteva and colleagues (322) who demonstrated that partial C4A or C4B gene deficiencies, and the loss-of-function exon 29 CT insertion were not risk factors predisposing to SLE in large United-Kingdom and Spanish cohorts. Further, there was no evidence that the presence of the HERV-K insertion in either C4A or C4B genes was associated with decreased serum C4 concentration in these Western Australian cohorts. This observation differs from earlier reports proposing that the presence of the HERV-K retroviral element decreases the rate of C4 transcription and therefore serum C4 concentration (318). Clinically low serum C4 concentrations can be explained by immune complex mediated activation of the classical complement pathway with subsequent loss of C4 protein in tissues where the rate of consumption is higher than that of synthesis (325). Low serum C4 concentrations in SLE patients may result therefore from ongoing consumption and hence represent a measure of autoimmune pathology.

This work confirms other reports that C4A genes are preferentially associated with the presence of the HERV-K retroviral element relative to C4B genes (with the absence of the insertion) (318). There were no significant differences between the SLE and control cohorts in mean number of C4A, C4B, C4A+C4B genes and C4 genes with and without the HERV-K insertion. Mean C4 concentrations in persons with long C4 genes, and short C4 genes, were not significantly different (see Figure A 7.6).

The positive correlation between C4A genes and the presence of the HERV-K insertion in the combined cohorts contrasted with the lack of correlation between

C4A genes and the absence of the HERV-K insertion. Correspondingly, the absence of the HERV-K insertion correlated positively with the C4B gene, and contrasted with absence of any significant correlation between C4B genes and the presence of the HERV-K insertion.

Previous reports (84, 323) have indicated the presence of a CT insertion in exon 29 which leads to non-functional C4A and C4B genes. Only one SLE patient (and no control persons) in this study was shown to have the CT insertion in exon 29. These data agree with the results of Boteva et al. (322), described above, and also of Puah and colleagues (326) who found no evidence for associations between SLE and the CT insertion (as well as three other known mutations in exons 13, and 20) in C4 genes in the Malaysian population.

Two independent methods were used to count C4 GCNs in all subjects tested. These were a commercial TaqMan™ assay that detected nucleotide sequences specific for either C4A or C4B and a custom quantitative PCR assay that detected the presence or absence of the ≈6.4kb retroviral HERV-K insertion in intron 9 of both C4A and C4B genes. Good agreement between both methods was observed for total C4 gene counts, although the total count by the HERV-K method showed a GCN dependent positive bias relative to the C4A & B total GCN. We reported previously that the TaqMan™ assay tends to underestimate total C4 GCNs when there are ≥ three C4A or C4B genes per person tested (86). Wu and colleagues reported similar observations (204) whereby at high GCN of C4A/ C4B/ C4 short and/or C4 long genes the TaqMan™ assay reported equivocal results with a propensity to underestimate GCN. Laboratory manipulation of DNA, including repeated freeze-thawing, may compromise the quality and quantity of DNA tested (86, 204) in these technically exigent assays, especially when high GCNs are present. Nevertheless, as shown by the Bland-Altman analysis, the bias in the data is relatively small (≈6%) and both assays were in complete agreement around median values for GCN.

It was of considerable interest that serum C4 concentrations in SLE patients and healthy controls correlated strongly with the C4A and C4B GCN. In SLE patients and healthy controls, approximately 88% and 99% respectively of the variability in serum

C4 concentrations could be attributed to C4 GCNs despite considerable individual variability in [C4]. This result indicates that for healthy persons expected serum C4 concentrations may be estimated from GCN. While a similar relationship exists in SLE patients, the co-existence of a pathological process consuming C4 may result in more variable serum [C4] such that GCN is not a reliable predictor of concentration for individual SLE patients, although, in conjunction with the serum [C4] expected reference ranges, it may still provide insights in disease management of SLE patients with well-managed disease. The results of this study are consistent with and supplement our earlier study of C4 GCNs in the WA Busselton population (86). A direct correlation between GCN and protein concentration in body fluids is the default expectation unless other mechanisms act to distort this relationship. For example, it has long been known that serum C2 concentrations are proportional to the number of expressed genes for this single dimorphic locus (327). Pouw and colleagues have also shown that the complement factor H-related protein 3 serum levels are determined by GCN variation at the CFHR3 locus (328). In this respect, the complex C4 genomic locus behaves similarly to other gene systems where protein synthesis correlates with GCN variation.

In conclusion, this report confirms the utility of serum [C4] in the diagnosis and management of SLE. Low C4 concentrations relative to C4 GCN are best explained by a mechanism involving consumption of C4 protein rather than decreased synthesis. Paradoxically, individuals manifesting complete deficiency in both C4A and C4B genes (a rare phenomenon (329)), and consequently lacking C4 protein, are also predisposed to SLE. Hence, in these individuals, the mechanisms resulting in the symptoms of SLE must exclude consumption of complement C4 protein. There is no evidence from this study that C4A GCN, the presence of the CT insertion, and/or the HERV-K insertion are significant risk factors for SLE.

5.5 Figures and Tables

5.5.1 Tables

Table 5.1: Summary table of the C4 quantitation data.

	SLE Patients		Healthy Controls		Significance
	Counts (%)	Mean (\pm SD)	Counts (%)	Mean (\pm SD)	
Serum [C4] (g/L)	56	0.1928 (0.1073)	33	0.2342 (0.0800)	$P = 0.0060$
C4A GCN	109 (50.4)	1.946 (0.7488)	61 (50.8)	1.848 (0.755)	NS
C4B GCN	107 (49.6)	1.911 (0.7926)	59 (49.2)	1.788 (0.4846)	NS
Total C4 GCN	216 (100)	3.857 (0.8186)	121 (100)	3.667 (0.7773)	NS
C4 Short GCN	64 (29)	1.143 (1.151)	29 (24)	0.8788 (0.9907)	NS
C4 Long GCN	155 (71)	2.768 (0.9907)	94 (76)	2.848 (1.326)	NS
HERV-K TOTAL GCN	219 (100)	3.911 (1.066)	123 (100)	3.727 (1.008)	NS

The table summarises the C4A, C4B, total C4, C4 short, C4 long and total HERV-K gene counts, mean and standard deviation values for SLE patients and controls. The table also indicates the serum [C4] means and standard deviations as well as the number of individuals (counts) in each group.

5.5.2 Figures

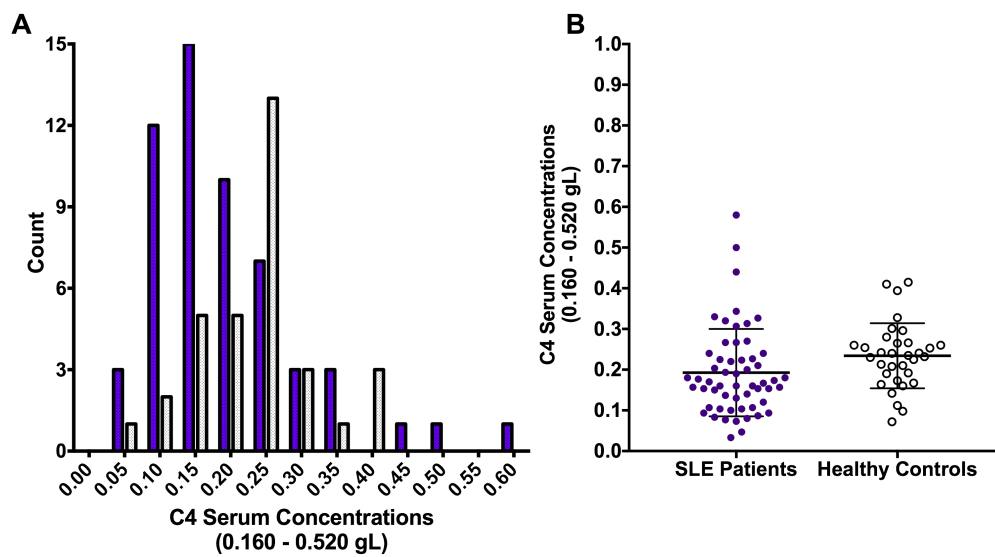


Figure 5.1: Serum[C4] in SLE patients and controls.

A: The figure shows the frequency distribution of the serum [C4] (X-axis) for the SLE patients (dark bars) and the control cohort (light bars). B: Distribution of [C4] showing the mean and standard deviation for each group; $p = 0.0060$ (Mann Whitney rank test). Mean concentrations: SLE patients 0.1928g/L (range 0.033 – 0.580); controls: 0.2342g/L (range 0.072 – 0.415). Median concentrations: SLE patients 0.1664g/L; controls 0.2350g/L. Serum C4 concentrations (0.160 – 0.520g/L) = reference ranges in the healthy population. 5.1.A “Count” refers to the number of individuals in each group.

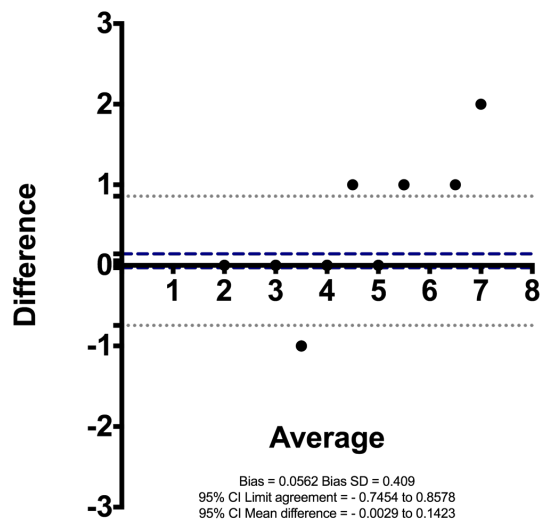


Figure 5.2: A Bland Altman analysis of two assay methods.

Bland Altman analysis plot of the difference versus average of the Taqman™ C4A + C4B and the Taqman™ Custom for HERV-K retroviral element in C4 genes assays. Plot shows the 95% confidence intervals (CI) of the mean difference (dashed lines) and 95% CI of the limit of agreement (dotted lines) between the two assays.

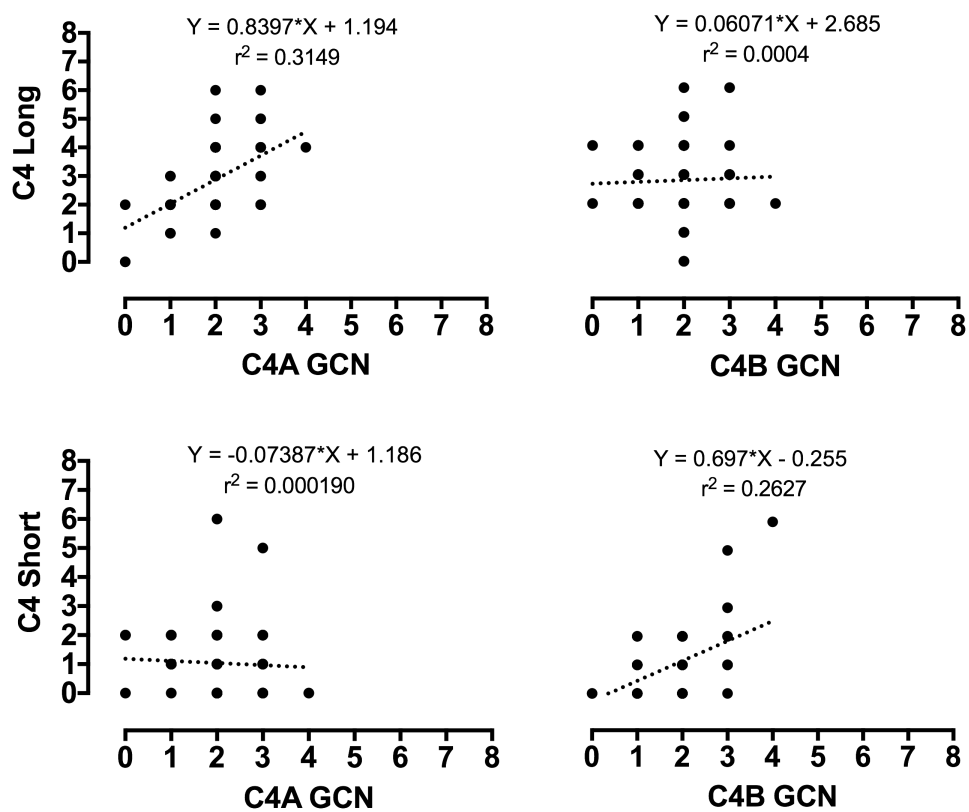


Figure 5.3: Relationship between C4 and HERV-K gene copy numbers.

Linear relationship between C4 long and C4A/ C4B genes (top graphs), and C4 short with C4A/ C4B genes (bottom graphs) for the 89 subjects in this study. Equations and r^2 values indicated on the graphs.

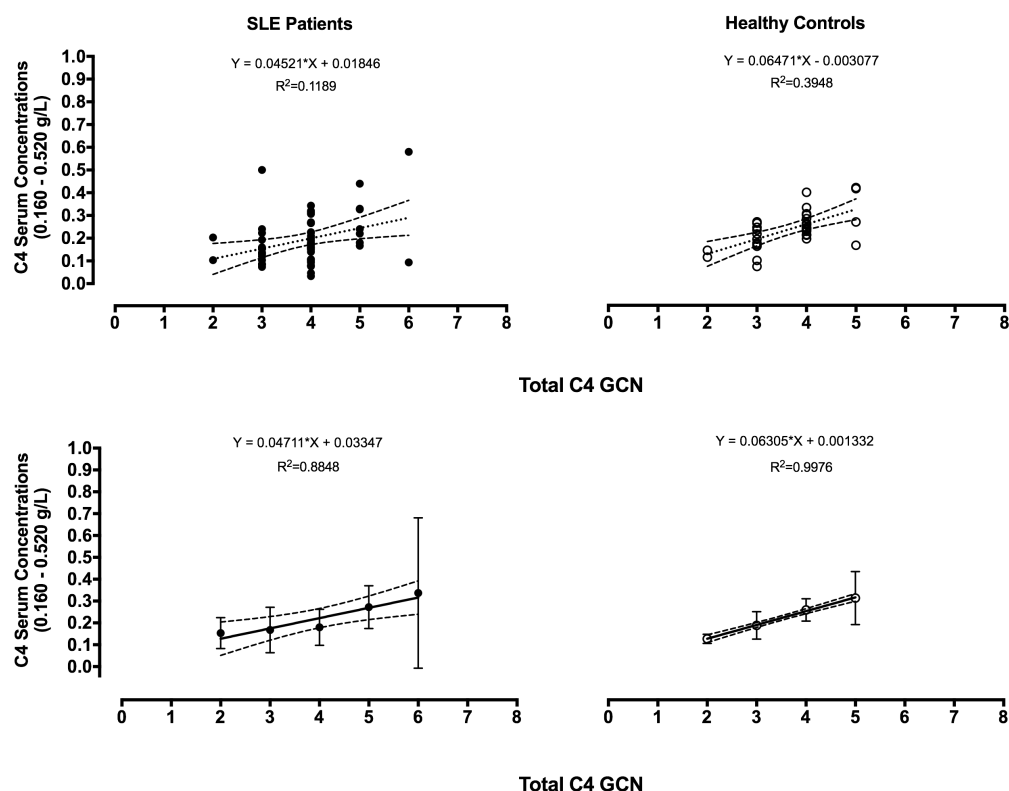


Figure 5.4: Serum [C4] stratified with C4 gene copy number.

The figure shows a stratification of the gene copy number for total C4 (X axis) with serum C4 concentrations (Y axis). The top graphs show the linear regression based on independent values whereas the bottom graphs are the mean values (aggregated) with standard deviation and 95% confidence intervals of the mean of the data. Serum C4 concentrations (0.160 – 0.520g/L) = reference ranges in the healthy population.

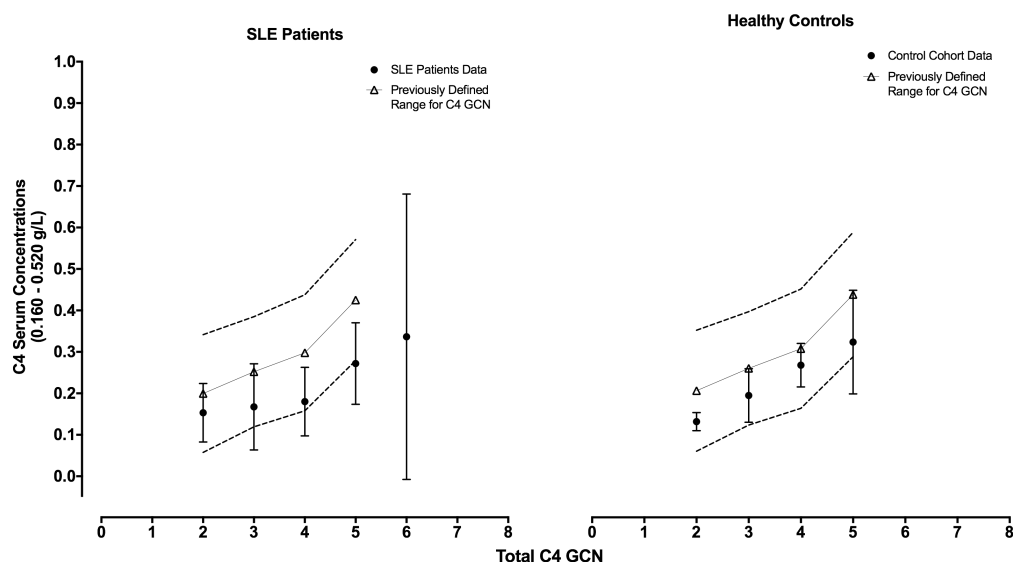


Figure 5.5: Previously defined serum [C4] ranges stratified with C4 gene copy number.

The figure shows a stratification of the total C4 gene copy numbers (X axis) for the SLE patients and control cohort (as indicated on the graph) with serum C4 concentrations (Y axis) as well as the previously defined normal reference ranges (86) for two, three, four and five GCN. Data shown indicate mean values for the SLE and control cohort, and mean values and standard deviations for the ranges previously described.

Chapter 6. Genetic Variation at the DNASE I Locus in an Australian Cohort of SLE Patients

The content of this chapter has been published in *Lupus: Open Access* (2017). Permissions for this article can be found in appendix B.1.4 and published article in B.2.3.

Margery-Muir A A, Wetherall J D, and Groth D M. Genetic Variation at the DNASE I Locus in an Australian Cohort of SLE Patients. *Lupus: Open Access* 6:130. 2017.

6.1 Abstract

Objective: DNASE I serum concentrations and activity, as well as sequence mutations have been implicated in the pathology of systemic lupus erythematosus (SLE). This study was undertaken to assess the serum DNASE I concentrations and assess the DNASE I sequence variation in a cohort of SLE patients and controls.

Methods: DNASE I serum concentrations were assayed in 56 SLE patients and 33 age and sex matched controls. All SLE patients and controls were genotyped for exomic alleles at the DNASE I locus and for the variable number tandem repeat alleles present in intron 4 (VNTR - *HumDN1*).

Results: Skewed DNASE I protein concentration distributions were observed with the mean value for SLE patients being 44.2 U/mL compared to 56.4 U/mL in controls (NS). No sample tested negative for DNASE I protein. Only two of the previously reported six exomic alleles (DNASE*1, DNASE*2) were identified, together with four VNTR alleles (three - six repeats). Both loci manifested Hardy-Weinberg equilibrium. Linkage disequilibrium was observed between exomic alleles and the VNTR alleles, especially between DNASE I*1 and four repeat VNTR (*HumDN1*) allele. No significant associations were observed between DNASE I concentrations and genotypes. Estimations of haplotype frequencies showed similar distributions for both SLE and the control cohorts, although it was noted that haplotypes containing DNASE*2 had an elevated frequency of longer VNTR alleles than did DNASE*1.

A meta-study of DNASE I exomic allelic frequencies showed similar frequencies to those obtained in other populations. For the VNTR locus, the longer alleles (five and six repeats) were more frequent, although there was no difference between SLE patients and controls.

Conclusion: This study does not support the hypothesis that specific two locus DNASE I genotypes predispose to SLE in the Western Australian cohort.

6.2 Introduction

Systemic lupus erythematosus (SLE) is an important systemic autoimmune disease characterised by the presence of autoantibodies binding double stranded DNA (henceforth dsDNA ab) and decreased levels of serum complement components C3 and C4 (290). It has been postulated that defective apoptotic clearance of immune complexes incorporating histone associated DNA predisposes to SLE (290). DNASE I, is an endonuclease (330) believed to facilitate the removal of cellular debris thereby preventing aberrant inflammation (331-333). There have been reports that DNASE I deficiency is a predisposing factor for SLE. Lower levels of DNASE I enzyme activity may be associated with lupus nephritis (20, 97) and correlate with disease activity state and clinical parameters (104). Yasuda and colleagues (330, 334-337) and Iida *et al.* (338) have identified exonic polymorphisms of the DNASE I gene, some of which appear to be associated with susceptibility to SLE (339). Furthermore, Koji and colleagues (1) have described an A-G transversion in exon two that results in defective synthesis of DNASE I in an SLE patient manifesting lower DNASE I activity. The DNASE I gene also contains a polymorphic 56bp variable number tandem repeat (VNTR – *HumDN1*) locus within intron four manifesting six alleles corresponding to molecular product sizes of 469, 525, 581, 637 and 693 for two, three, four, five and six repeats respectively (340, 341). Yasuda and colleagues (340) reported that the three repeats allele of VNTR (*HumDN1*) was in linkage disequilibrium with DNASE I*1 allele whilst the VNTR alleles four and five were in linkage disequilibrium with DNASE I*2.

In this study, genetic polymorphisms at both the DNASE I locus and at the VNTR (*HumDN1*) locus were typed in a Western Australian population of SLE patients and controls with known serum concentrations of DNASE I protein. The genetic diversity of DNASE I polymorphisms in these cohorts was compared with other studies and associations sought with disease and serological parameters indicative of SLE pathology.

6.3 Results

6.3.1 DNASE I protein concentrations in SLE patients and controls.

DNASE I protein concentrations observed in serum samples of SLE patients and controls are shown in Figure 6.1. The distribution of concentrations in both cohorts was skewed; the mean concentration of DNASE I protein in SLE patients was lower than that of the controls (44.2 U/mL versus 56.4 U/mL) although the difference was not significant. A preliminary investigation of associations between DNASE I serum concentrations and co-morbidities and drug therapies in the SLE patients was undertaken using heat maps is shown in Figure 6.2C and Figure 6.2D respectively.

6.3.2 Allele and genotype frequencies at the DNASE I and intron 4 VNTR (*HumDN1*) loci.

Allele frequencies at the DNASE I exomic and VNTR (*HumDN1*) loci are summarised in Table 6.2. Alleles at both intragenic loci exhibited Hardy-Weinberg equilibrium and linkage disequilibrium. The frequencies of haplotypic combinations at both the DNASE I and VNTR (*HumDN1*) loci are shown in Figure 6.3. Haplotypes with VNTR alleles of 4, 5 and 6 repeats were more prevalent in both the SLE and control cohorts.

6.3.3 Ethnic variation in DNASE I polymorphisms

The allele frequencies reported in this study are in general agreement with the metastudy of ethnic allele frequency variation shown (see Table 6.3). Only the Ovambos' of African origin have higher frequencies of the DNASE I*1 relative to the DNASE I*2 allele. No apparent significant differences between DNASE I allele frequencies were observed for either the SLE patients or the healthy controls. A comparison of ethnic variation in the frequencies of the internal VNTR (*HumDN1*) locus within the different ethnic groups is shown in Table 6.4. The frequencies of the six alleles reported in this study were similar to those observed in other population with the exception that the long six repeat allele was more frequent in both the control and SLE populations reported herein. The genetic differentiation of the

Ovambo and Japanese populations was evident with the three-repeat allele being more prevalent than in the other populations reported.

6.3.4 Are serum DNASE I concentrations dependent on genotype?

DNASE I serum concentration distributions are shown ranked by genotype in Figure 6.4.

There were no significant associations between DNASE I serum concentrations and either the DNASE I exomic genotypes or the repeat number variation of the VNTR (*HumDN1*) locus. The lowest concentration levels of serum DNASE I were associated with DNASE I*2 genotype, and with the higher number of repeats at the VNTR (*HumDN1*) locus.

6.3.5 Associations between serological parameters and DNASE I and VNTR (*HumDN1*) alleles.

A correlation matrix was generated for age, ethnicity, (C1q), (C3), [C4] and (DNASE I) versus VNTR (*HumDN1*) and DNASE I alleles (Table A 7). No significant correlations between the various parameters were observed except for serum concentrations of DNASE I and C1q complement protein ($r = 0.653$; $p = 0.05$). In the SLE cohort, the VNTR (*HumDN1*) repeat number was significantly correlated with (C3) and [C4] (0.304 ; $p = 0.03$ and 0.279 ; $p = 0.04$ respectively). However, specific alleles at both the VNTR (*HumDN1*) and DNASE I loci were significantly correlated in the SLE and control cohorts ($r = 0.760$; $p = 0.0001$; 0.569 ; $p = 0.0005$ respectively) (Table A 8 & Table A 9).

In the absence of well-defined associations between specific DNASE I genotypes and SLE, heat maps were used to visualize trends in disease severity that may be related to DNASE I genotypes or serum concentrations in both the SLE patient and control cohorts. The heat map analyses (Figure 6.2) show qualitatively that SLE patients with higher numbers of VNTR (*HumDN1*) repeats (≥ 5) had lower serum concentrations of DNASE I protein and required more intensive drug regimens for management of their disease than persons with low numbers of repeat motifs.

6.4 Discussion

In this investigation only the two most frequent (ie. DNASE I*1 and DNASE I*2) of the six exonic alleles defined at the DNASE I locus were observed, although the typing system (shotgun sequencing) would have detected them if present. No variations in the nucleic acid sequence within exons two, five and seven were present in any of the individuals tested. The remaining four alleles (DNASE I*3, *4, *5 and *6) are rare and there are few reports of their frequencies in the other populations (342). The frequencies of the DNASE I*1 and 2 in persons of European and Asian ethnicity were similar to previously published reports (342-344). Four alleles were observed at the VNTR locus with the number of repeats varying from three to six. A greater frequency of the longest six repeat allele was observed in both cohorts in this study which may reflect improved typing techniques. Only in the Ovambo and Ghanian populations did allele frequencies at both loci differ markedly from the values reported in other populations. This is consistent with an ancestral version of the DNASE I gene being present in modern day African populations (345).

Allele frequencies at both the DNASE I and VNTR (*HumDN1*) loci manifested Hardy-Weinberg equilibrium despite the relatively small sample size of the populations typed. As would be expected, both intragenic loci exhibited linkage disequilibrium (LD) as has been previously reported (340, 346).

Of the 36 possible genotypes comprising observed alleles at these two loci, 13 were observed in the SLE patients and 12 in the control group. These data demonstrate the presence of a restricted number of haplotypes in our populations. AlFadhli and co-workers have shown that higher number of the VNTR (*HumDN1*) repeats are a risk factor for SLE and that the allele with five repeats was associated with a reduction of DNASE I enzyme activity (341). It was not possible to measure the DNASE I enzyme activity, in this study however serum protein DNASE I concentrations were assayed in both populations. All persons tested exhibited DNASE I protein in their serum. Significant differences in serum DNASE I protein concentrations between SLE patients and the control cohort were not observed, although the mean concentration in the SLE cohort was slightly lower than in the control group. If DNASE I activity is

proportional to the concentration of DNASE I protein it is unlikely that non-expressed mutant genes were present in the cohorts investigated.

Associations between the VNTR (*HumDN1*) locus and complement C3 and C4 serum concentrations were observed in the SLE cohort but not in the control group. Hence, the number of VNTR (*HumDN1*) repeats in the SLE patients may influence clinical parameters. AlFadhli and co-workers have shown that higher number of the VNTR (*HumDN1*) repeats are a risk factor for SLE, and moreover, that the five-repeat allele was associated with a reduction of DNASE I enzyme activity (341).

The heat map analysis (Figure 6.2) showed qualitatively that SLE patients with higher numbers of VNTR (*HumDN1*) repeats (\geq five) had lower serum concentrations of DNASE I protein than the controls and required more intensive drug regimens for management of their disease. However, quantitative analysis did not reveal any significant correlations between the serum concentrations of DNASE I protein and either phenotypes at the DNASE I and VNTR (*HumDN1*) loci or with disease severity (see Figure 6.3). Other reports (341, 347), in particular that of AlFahdi (341), show that a high number of VNTR repeats are a risk factor for SLE and may cause decreased DNASE I activity. Positive correlations between DNASE I and C1q serum concentrations and the length of the VNTR allele with serum concentrations of complement components C3 and C4 were observed and noted. If low DNASE I activity and/or concentration impairs removal of apoptotic material thereby predisposing to SLE, it might be expected that VNTR alleles negatively correlate with C3, C4 and C1q serum concentrations in SLE patients. Hence this report does not support the hypothesis that specific two locus DNASE I genotypes predispose to SLE in the Western Australian cohort.

6.5 Tables and figures

6.5.1 Tables

Table 6.1: DNASE I polymorphisms in exons 2, 5, 6, 7 & 8.

DNASE I allele	Exon 2	Exon 5	Exon 6	Exon 7	Exon 8
DNASE I*1	CAG – Gln	GTG – Val	CCC – Pro	CGC – Arg	CAA – Gln
DNASE I*2	CAG – Gln	GTG – Val	CCC – Pro	CGC – Arg	C <u>G</u> A – Arg
DNASE I*3	CAG – Gln	GTG – Val	<u>G</u> CC – Ala	CGC – Arg	CAA – Gln
DNASE I*4	<u>G</u> AG – Glu	GTG – Val	CCC – Pro	CGC – Arg	C <u>G</u> A – Arg
DNASE I*5	CAG – Gln	<u>A</u> TG – Met	CCC – Pro	CGC – Arg	C <u>G</u> A – Arg
DNASE I*6	CAG – Gln	GTG – Val	CCC – Pro	<u>T</u> GC – Cys	C <u>G</u> A – Arg

Nucleotide/amino acid substitutions underlying the major DNASE I gene polymorphisms as summarised by Fujihara and colleagues (18).

Table 6.2: Allele frequencies of the DNASE I and VNTR (*HumDN1*) loci.

Population	DNASE I*1	DNASE I*2	VNTR (<i>HumDN1</i>) 3	VNTR (<i>HumDN1</i>) 4	VNTR (<i>HumDN1</i>) 5	VNTR (<i>HumDN1</i>) 6
SLE (n=56)	0.393 (n=44)	0.607 (n=68)	0.036 (n=4)	0.304 (n=34)	0.366 (n=41)	0.295 (n=33)
Control (n=33)	0.394 (n=26)	0.606 (n=40)	0.000	0.409 (n=27)	0.348 (n=23)	0.242 (n=16)

Allele frequencies for the DNASE I locus and the intron 4 VNTR (*HumDN1*) locus in Australian SLE patients and healthy controls (numbers in brackets indicate the number of alleles in each group).

Table 6.3: Frequency data of the DNASE I*1 & *2 from published reports.

Population (References)	Allele DNASE I*1 Not diseased	Allele DNASE I*2 Not diseased	Allele DNASE I*1 SLE	Allele DNASE I*2 SLE
Mixed Europeans (this study) Controls (n = 25) (SLE n = 24)	0.380	0.620	0.375	0.625
Mixed Asians (this study) Controls (n = 5) SLE (n = 11)	0.500	0.500	0.454	0.545
Mixed other (this study) Controls (n = 3) SLE (n = 21)	0.333	0.667	0.381	0.619
Germans (26; 27)	0.294	0.705		
Germans (28)	0.265	0.728		
Spanish (29)	0.3003	0.6997	0.2246	0.7754
Japanese (26)	0.547	0.445		
Japanese (28)	0.595	0.405		
Koreans (28)	0.597	0.403		
Chinese (Han) (30)	0.536	0.464		
Chinese (Shenyang) (31)	0.5670	0.4330		
Chinese (Guangzhou) (31)	0.5417	0.4583		
Argentineans (14)	0.280	0.720	0.340	0.660
Turks (18)	0.221	0.779		
Ovambos (18)	0.872	0.128		
Ovambos (32)	0.872	0.128		
Ovambos (28)	0.889	0.111		
Indian Tamils (33)	0.520	0.490	0.495	0.505

Frequency data for the DNASE alleles *1 and *2 are derived from published reports. The rare DNASE I*3; *4; *5 and *6 alleles are not included due to insufficient published data. "Mixed other" control group contained individuals of Middle Eastern and Indian ethnicity whereas the "Mixed other SLE" group included individuals from African, South American and Australian Indigenous ethnicities which were grouped together as there were only a small number of individuals in each.

Table 6.4: VNTR (humDN1) repeat frequencies from published reports.

Population (References)	Allele 1 Not diseased	Allele 2 Not diseased	Allele 3 Not diseased	Allele 4 Not diseased	Allele 5 Not diseased	Allele 6 Not diseased	Allele 1 SLE	Allele 2 SLE	Allele 3 SLE	Allele 4 SLE	Allele 5 SLE	Allele 6 SLE
Mixed Europeans (this study) controls (n = 50) SLE (n = 48)	0.000	0.000	0.000	0.380	0.340	0.280	0.000	0.000	0.000	0.250	0.438	0.312
Mixed Asians (this study) controls (n = 10) SLE (n = 22)	0.000	0.000	0.000	0.600	0.400	0.000	0.000	0.000	0.045	0.409	0.364	0.182
Mixed other (this study) controls (n = 6) SLE (n = 42)	0.000	0.000	0.000	0.167	0.500	0.333	0.000	0.000	0.071	0.309	0.286	0.333
German (16)	0.000	0.010	0.264	0.337	0.393	0.006						
German (34)	0.000	0.000	0.390	0.329	0.280	0.000						
Japanese (16)	0.000	0.010	0.556	0.271	0.162	0.001						
Japanese (35)	0.000	0.026	0.583	0.211	0.180	0.000						
Chinese (35)	0.000	0.021	0.474	0.300	0.205	0.000						
Turks (35)	0.000	0.000	0.236	0.366	0.398	0.000						
Kuwaiti (17)	0.000	0.011	0.332	0.391	0.163	0.103	0.000	0.026	0.270	0.257	0.447	0.000
Kuwaiti (19)	0.000	0.020	0.310	0.370	0.180	0.120	0.000	0.050	0.280	0.260	0.420	0.000
Ovambo (35)	0.000	0.087	0.788	0.120	0.005	0.000						
Ghanaian (34)	0.005	0.083	0.771	0.094	0.047	0.000						
Iranian (36)	0.000	0.000	0.322	0.411	0.208	0.058	0.000	0.000	0.251	0.408	0.279	0.061

Frequency data for the VNTR (humDN1) number of repeats are derived from referenced published reports. The rare DNASE I*3; *4; *5 and *6 alleles are not included due to insufficient data. "Mixed other" control group contained individuals of Middle Eastern and Indian ethnicity whereas the "Mixed other SLE" group included individuals from African, South American and Australian Indigenous ethnicities which were grouped together as there were only a small number of individuals in each.

6.5.2 Figures

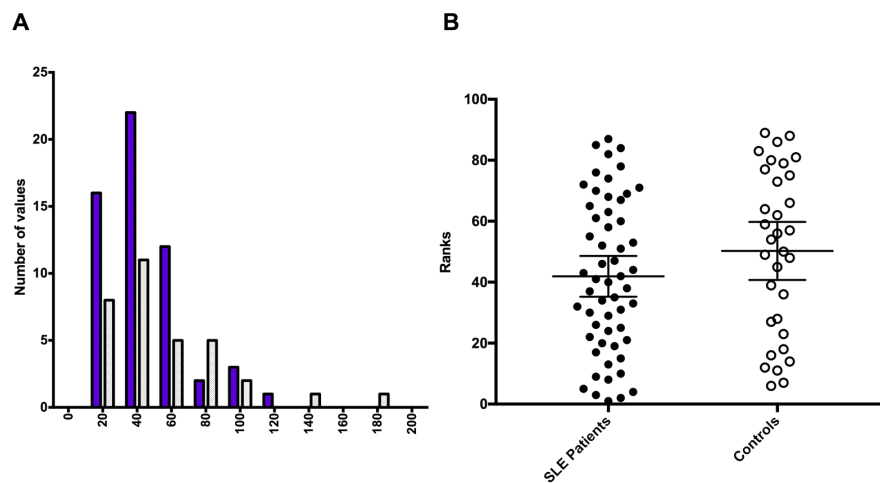


Figure 6.1: Serum DNASE I concentrations.

A: dark bars - the frequency distribution (U/mL) in the SLE (mean = 44.2 U/mL) and light bars - control (mean = 56.4 U/mL) populations. B: Ranks of serum DNASE I concentrations in SLE and control cohorts showing means \pm standard deviation – not significantly different.

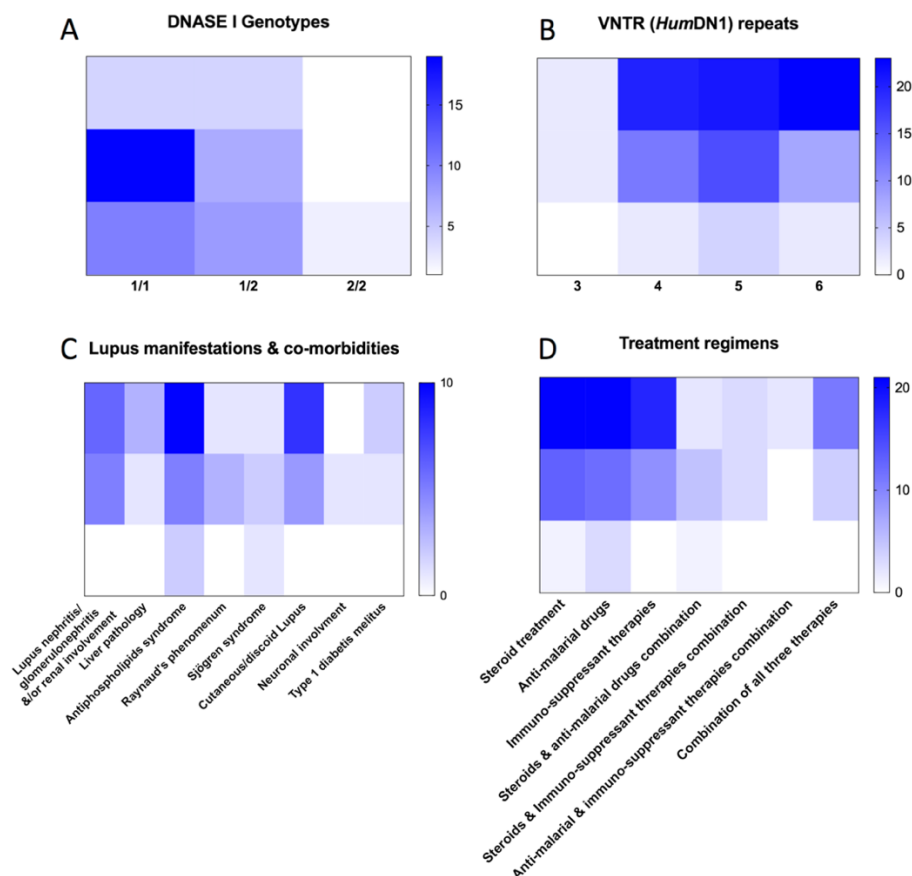


Figure 6.2: Heatmaps for the SLE patients.

Heatmaps showing associations between mean concentrations of serum DNASE I in SLE patients stratified in three groups defined as low concentration range, mid-concentration range and high concentration range with mean concentrations of DNASE I = 29U/mL top row, DNASE I = 59U/mL middle row, and DNASE I = 103U/mL bottom row respectively. Figures 2A and 2B: DNASE I concentrations versus DNASE I genotypes and VNTR (*HumDN1*) repeats genotypes respectively. Figures 2C and 2D: DNASE I concentrations in SLE patients versus co-morbidities and drug therapies respectively.

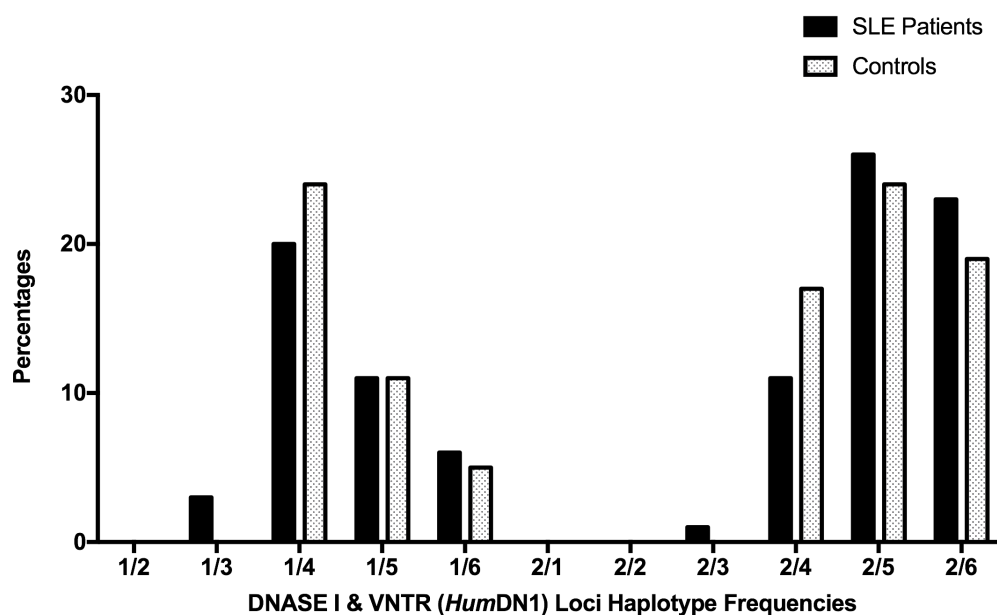


Figure 6.3: DNASE I (left number) & VNTR (*HumDN1*) (right number) Loci Haplotypes.
The figure shows percentage haplotype frequencies in both loci for the SLE patients and control cohort.

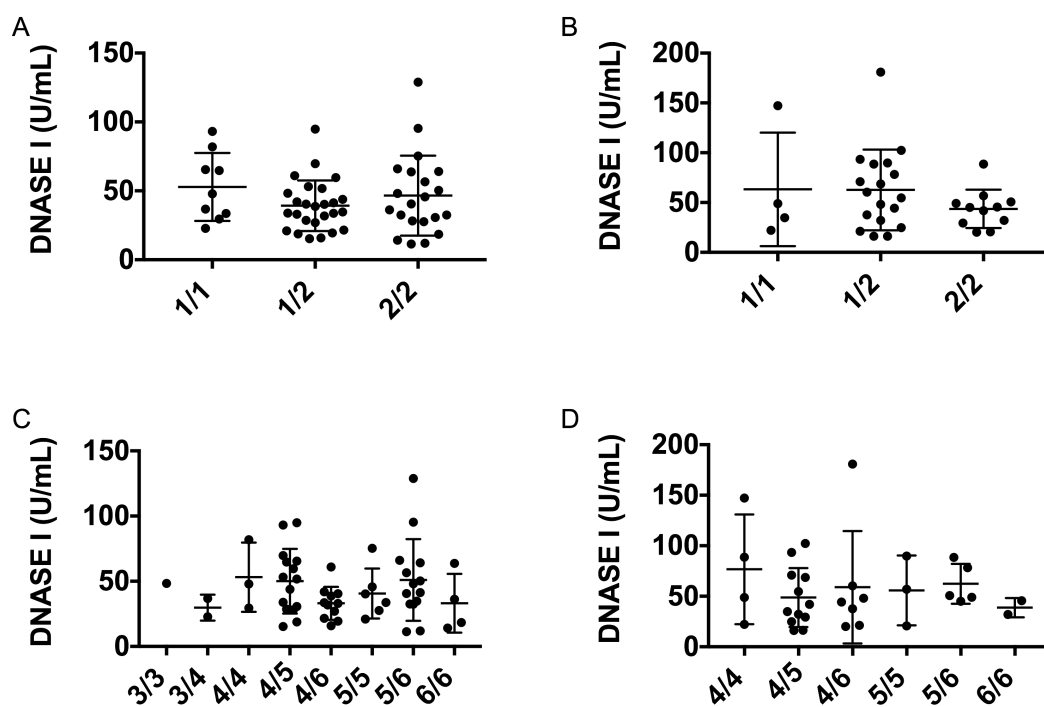


Figure 6.4: Serum [C4] associated with DNASE I and VNTR (*HumDN1*) phenotypes.
The figure shows the mean concentrations (U/mL \pm SD) of the DNASE I protein in SLE patients (A & C) and controls (B & D) associated with DNASE I phenotype DNASE I*1 or DNASE I*2 (A and B) and with the VNTR (*HumDN1*) number of repeats (C and D).

Chapter 7. Final discussion and concluding comments

7.1 Discoveries and Discussion

7.1.1 Introduction to the hypothesis

Systemic lupus erythematosus (SLE) is a polygenic, multifactorial autoimmune disease, characterised by autoantibody production, immune complexes and inflammation leading to multiple organ and tissue damage. Recently, it has been proposed that defective clearance of apoptotic materials generates inflammatory mechanisms manifesting as SLE in genetically predisposed individuals. This project focused on the investigation and interpretation of a panel of laboratory assays used to diagnose and management SLE with this hypothesis as a guide for mechanism(s). It is anticipated that the results obtained will provide clinicians with valuable insights for diagnosing, treating and monitoring SLE patients in Western Australia.

Characteristics of SLE pathology that define this multisystem autoimmune disease were identified in this project; specifically, it was proposed that underlying immunopathology and genetic predisposition should be reflected in the laboratory assays used in the diagnosis and management of SLE. The review of contemporary literature centred therefore on the conventional female preponderance of SLE, and included a refinement of its epidemiology, together with the role of genetic deficiencies in the predisposition to SLE. Moreover, contemporary relevant SLE research on twins, sex chromosomal abnormalities, genome wide association studies and possible environmental factors were investigated.

Mechanisms underlying the pathology of SLE have recently been reviewed in a comprehensive publication by Tsokos and co-workers (344). Despite a wide body of information available in the form of published articles, reviews and books readily available via institutions, the multiple potential aetiologies of SLE still remain frustratingly unresolved. Based on the defective apoptosis processes hypothesis, we generated a mechanistic model that formed the basis of the potential cellular,

serological and genetic pathways that were investigated in the practicum of this project. Details of this model are summarised in Figure A 7.2.

7.1.2 Important outcomes of the project

Outcomes from this project that convey new information relevant to the laboratory based diagnosis and management of SLE, or which imply mechanistic information are summarised below.

- ❖ Quantitation of IL-17 and Th17 cells in treated SLE patients is not justified. Future research will be required to confirm the relevance and potential role of $\gamma\delta$ T cells and the mDC2 cells in both newly diagnosed and treated SLE patients.
- ❖ Quantitation of α C1q ab in serum is not a useful assay for the diagnosis of SLE, however it is a useful assay for the exclusion of a diagnosis of SLE. Anti-C1q ab levels should be investigated further for their utility in monitoring SLE patients for disease activity.
- ❖ C4 gene copy number can be estimated by two real time PCR quantification methods. These are a commercial TaqMan[®] assay detecting a five-amino acid difference between C4A and C4B genes in exon 26, and a custom assay detecting the presence or absence of a retroviral HERV-K element in intron nine of C4 genes. Both assays agreed well, but the C4A and C4B specific assay is more practicable for investigative use.
- ❖ Concentrations of C4 protein in serum correlate with total C4 GCN (ie both C4A and C4B genes) in human populations, but variance, especially in SLE patients, makes prediction of individual serum C4 concentrations from C4 GCN unreliable, except in rare instances of C4 gene deficiency. Hence, despite the complexity of the complement C4 genomic region, with an average of four C4A and B genes per individual, the synthesis of C4 protein behaves in the same manner as other conventional protein synthesising genes for which serum concentration reflects gene copy number (see also Chapter 5).

- ❖ Low C4 serum concentrations in SLE would appear to be the result of consumption rather than defective synthesis. In this study low C4A or C4B GCNs are not risk factors for SLE. Furthermore, deficient synthesis due to the rare frameshift CT insertion in C4 genes, nor the presence of the HERV-K retroviral element does not appear to contribute significantly to serum C4 concentrations.
- ❖ Assays for the presence of either the CT frameshift insertion, or the presence of the HERV-K retroviral element, in C4 genes is not useful in the diagnosis and monitoring of SLE.
- ❖ Specific genotypes at the polymorphic DNASE I locus are not associated with SLE, and serum concentrations of DNASE I are not useful in the diagnosis of SLE.

7.1.3 Commentary

It was expected that IL17 concentrations and proportions of Th17 cells would be increased in managed SLE patients. In contrast, we found very low frequencies in the SLE cohort, and synthesis of IL17 was increased in $\gamma\delta$ T cells as opposed to Th17. We also found a reversal in the proportions of mDC1 & mDC2 in SLE patients, and mDC2 correlated with the frequencies of the $\gamma\delta$ T cell subset. In SLE patients, we found a relative increase in serum concentrations of inflammatory cytokines IFN γ TNF α , MCP-1, IL-6, IL-8 and IL-18, indicating inflammatory signatures despite treatments. Low dose prednisolone (349), hydroxychloroquine, azathioprine, methotrexate and mycophenolate mophetil are widely used drug treatments for SLE (350, 351). Anti-malarial treatments exert their effect via multiple molecular pathways that include anti-thrombic, anti-lipidic and anti-inflammatory (352). Plasmacytoid dendritic cells producing IFN α promoting B and T cell proliferation are targeted by anti-CD20 therapies (353). Mycophenolate mofetil regulates CD4 $^{+}$ T cell polarisation by inhibiting the expression of CD40L (354). Hence, these treatments should suppress plasmacytoid DCs, preventing T cell activation and further differentiation of Th17 cells. We suggest therefore that the drug therapies of the SLE patients investigated

suppressed TH17 cell numbers, while permitting a $\gamma\delta$ T cell/mDC2 axis to maintain ongoing inflammations

Defective apoptosis is accompanied by binding of complement C1q to apoptotic debris with subsequent α C1q ab generation. It was observed that α C1q ab levels were elevated in SLE patients compared to controls, with all individuals tested manifesting some, albeit below the advocated cut-off level recommended by the manufacturer. In addition, quantitation of α C1q ab serum concentrations demonstrated that groups of SLE patients can be identified based on their concentrations levels, which are associated with specific co-morbidities (i.e. renal syndrome) and combined treatment regimen comprised of steroids, anti-malarial and immune-suppressant therapies. The levels of serum α C1q ab correlated with dsDNA antibody but not with serum C1q protein levels in the SLE patients. Measurement of α C1q ab is not advocated for diagnosis of SLE, but is certainly useful for its exclusion.

Quantitation of serum protein levels of complements C1q, C2, C3 and C4 are used in routine testing for the monitoring of SLE as autoantibodies generated in active disease leads to activation and consumption of the classical pathway complement proteins, which is reflected in the low serum levels of these components. In our study, low C4 serum protein concentrations were clearly of diagnostic importance for SLE. However, deficiencies of complement proteins C1q, C2 and C4 remain as anomalous well-known risk factors for SLE for which the mechanism(s) remain elusive.

It was shown that for both the SLE and control cohorts the concentrations of serum C4 proteins were correlated with GCN, but variation in individuals precluded it usefulness in predicting one from the other. Consumption of C4 in tissues is probably the mechanism by which the serum concentrations remain low despite treatments in some individuals, rather than deficient synthesis due to either lower C4A and/or C4B GCN. Furthermore, these results did not support low C4A or C4B GCN as a risk factor for SLE, although this view is often advocated (81). Contrary to published reports

(318, 320), the HERV-K retroviral element had no effect on the serum C4 protein synthesis, nor was the CT insertion associated with SLE.

Endonucleases such as DNASE I are important for the degradation of apoptotic material (330). Hence, defective DNASE I activity/ protein synthesis would imply a significant risk factor for SLE by way of deficient clearance of apoptotic debris.

Decreased DNASE I concentrations in patients relative to controls were not observed and similar levels in diseased and non-diseased groups imply a functioning enzyme in both. In addition, the typing of polymorphisms and genotypes identified at the DNASE I locus indicated no association with SLE pathology, although individuals with a deficiency of DNASE I are associated with symptoms of SLE.

Based on the above results, we propose a variation of this mechanistic process by which, in the presence of treatment regimens, mDC2 and $\gamma\delta$ T cells recognise apoptotic material and cooperate leading to the synthesis of intracellular IL17 by $\gamma\delta$ T cells, resulting in chronic inflammation. Irrespective of DNASE I enzyme status, apoptotic material is recognised and targeted by C1q molecules, which induces the production of α C1q ab in the kidneys of SLE patients. Bound C4 and α C1q ab to ICs and synthesis of intracellular IL17 are events which result in chronic inflammation and tissue damage. For the convenience of the reader an updated version of our proposed mechanisms (Figure A 7.2) is now available in Figure 7.1.

7.2 Caveats

In the recruitment of patients, we were reliant on generous colleagues and clinicians from the hospitals involved. It was extremely beneficial to have direct contact with them. This enabled a clear understanding of the processes involved in the monitoring and treatment of patients. However, we found that hospital records did not report the same information consistently, resulting in incomplete data for some patients. Furthermore, hard copies of the patients' records varied considerably, often containing disparate information which frustrated comparisons. The digitisation of medical records, which is gaining pace in developed nations, will reduce this type of problem in future studies.

With the advantage of hindsight, it became clear that working with SLE patients that included only diagnosed and treated individuals imposed some limitations on the interpretation of results. At the commencement of the project it was expected that some of the cohort would comprise newly diagnosed SLE patients, but unfortunately this did not eventuate. In addition, due to ethical, funding constraints and time limitations, we were unable to collect blood samples sequentially for longitudinal studies.

In retrospect, the cellular studies should have included an assay for mediators of apoptosis, together with additional cluster of differentiation and activation markers for characterisation of DCs and T cells. However, these were not included initially and there was no opportunity for repeating many of these flow cytometry based assays. In similar vein, it was planned to assay DNASE I activity as well as DNASE I protein concentration. However, the late withdrawal from the market of commercial kits for this assay prevented this outcome.

Many differences were observed throughout this project between the test cohort of SLE patients and the control group. The interpretation of these differences was initially determined by estimating a “*p*-value” relative to the null hypothesis, usually that there is no real difference between groups. As the project progressed, it was clear that this simplistic approach to identifying a discovery was unsatisfactory and should no longer be used (for example, see editorial comment, Nature 551, page 557 30/11/17). A *p*-value represents a statement of the probability that the differences observed are real. However, estimating the probability of a false discovery is also equally important in concluding whether a “real” discovery has been observed. Hence, many research publications now require a more rigorous interpretation of research data. Consequently, in this thesis and cognate publications, we have reported conventional *p* values for all “results”; however only those results with *p* values less than 0.001 are interpreted as possible ‘discoveries’ that should in general terms be reproducible. The availability of complex datasets incorporating genomic sequences and other aspects of bioinformatical research increases the necessity for more rigorous statistical evaluation of numerical data.

7.3 The Forward View

Over recent decades the diagnosis and management of SLE has improved dramatically. Mortality from SLE has declined largely due to the use of immunosuppressive and anti-inflammatory drugs, and improved laboratory medicine to monitor and diagnose this multifaceted autoimmune disease. It is noteworthy that juvenile SLE is still associated with a high mortality rate and overall, cures remain elusive. Despite intensive research efforts over past decades, the aetiology of SLE remains incompletely understood. Indeed, it seems likely that the pathology of SLE derives from aberrant inflammatory responses arising from multiple distinct mechanisms in genetically predisposed individuals. Although the mechanism of deficient apoptosis explored in this project has been useful in interpreting outcomes, this hypothesis will not explain all occurrences of SLE.

With the advantage of hindsight, identifying genetic and environmental factors that predispose to SLE seem important topics for future research. The incidence of SLE in most communities seems to have remained relatively stable over many years. Hence, it seems that more research to identify genetic factors that predispose to SLE are likely to provide new knowledge clarifying mechanistic pathways in SLE with the potential for more curative treatments. The advent of affordable whole genome sequencing together with sophisticated software for interrogating sequence databases opens a door to exciting progress for many diseases including SLE. The impressive discordance between both monozygotic and dizygotic twins requires an explanation that will probably answer many important questions relating to the role of both genetic and environmental factors in the pathology spectrum of SLE. In our opinion therefore, a comparison of genomic sequences of both monozygotic and dizygotic twins, for which at least one twin is diagnosed with SLE, will be a productive way forward in identifying the factors that contribute and/or predispose to SLE in humans. Such twins, although relatively rare, occur sufficiently frequently that creating a national or international database is possible. This approach will be more informative, than extending genome wide association studies that are open to many levels of interpretation. The way ahead is exciting and holds the promise of cures rather than treatments.

7.4 Figures

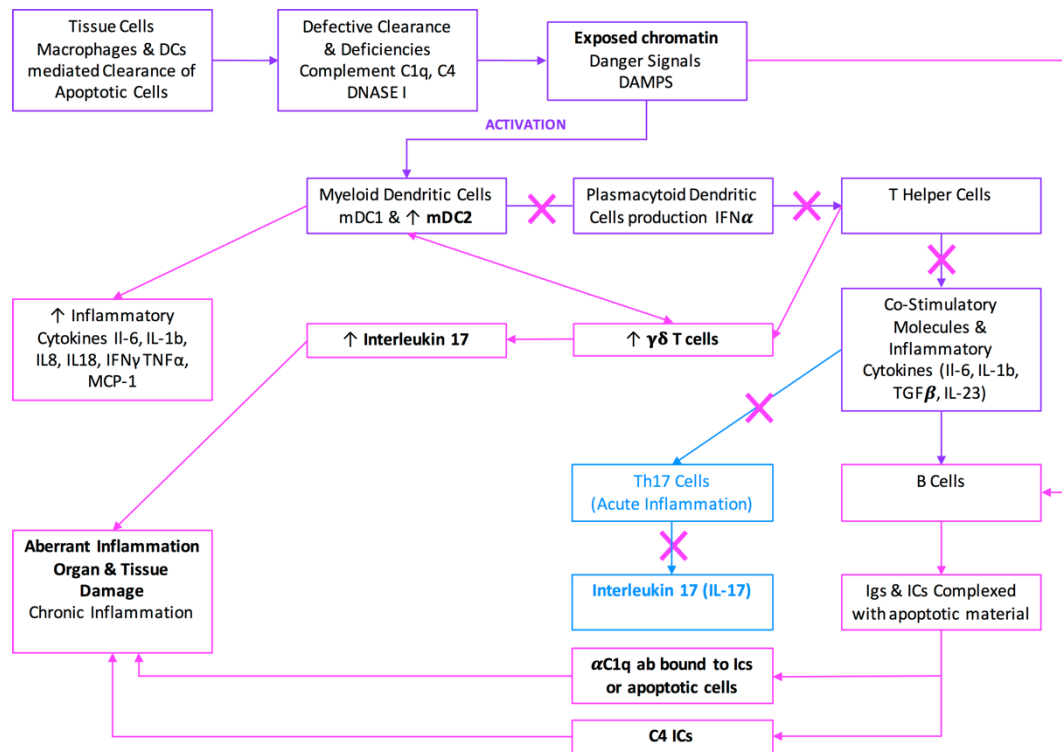


Figure 7.1: The figure shows the final adjusted mechanisms pertaining to SLE pathology in this cohort.

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Appendix A List of figures and tables

A.1 Tables

A.1.1 Chapter 2 tables

Table A 1: Serology parameters for the SLE patients

	Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Mean	Std. Deviation	Lower 95% CI of mean	Upper 95% CI of mean
Age at time of collection	56	19	34	48	58.25	77	46.55	14.91	42.56	50.55
Clq (g/L) (0.21 - 0.39)	56	0.13	0.28	0.31	0.36	0.46	0.32	0.07	0.31	0.34
C3 (g/L) (0.88 - 1.98)	56	0.33	0.76	0.95	1.14	1.74	0.96	0.27	0.89	1.03
	56	0.03	0.11	0.17	0.24	0.58	0.19	0.11	0.16	0.22
Anti-Clq (U/mL)	56	3.19	8.58	24.74	72.24	371.20	54.70	77.37	33.98	75.42
CRP (g/L) (<5m)	48	1	1.19	2.95	8.41	42.00	6.11	8.37	3.68	8.54
Ds-DNA ab (IU/mL) (<7)	55	1	4	7.70	17	243.30	19.39	35.74	9.73	29.05
ANA	21	5	20	30	30	30	24.13	7.48	20.72	27.53
DNASE I (U/mL)	56	11.37	28.27	39.45	58.86	128.80	44.17	23.95	37.76	50.58
CD40LG (pg/mL)	56	500	4371	6984	11093	25362	8015	5225	6616	9414

Table A 2: Serology parameters for the control cohort.

	Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Mean	Std. Deviation	Lower 95% CI of mean	Upper 95% CI of mean
Age at time of collection	33	21	27.5	48	56	75	44.77	17.12	38.73	50.9
C1q (g/L) (0.21 - 0.39)	33	0.24	0.28	0.31	0.34	0.41	0.31	0.04	0.29	0.33
C3 (g/L) (0.88 - 1.98g)	33	0.94	1.12	1.39	1.57	2.27	1.41	0.29	1.30	1.51
C4 (g/L) (0.160 - 0.520)	33	0.07	0.18	0.23	0.26	0.41	0.23	0.08	0.20	0.26
Anti-C1q (U/mL)	33	3.85	5.37	7.30	8.85	109.90	12.98	20.71	5.64	20.33
DNASE I (U/mL)	33	16.11	30.75	48.14	74.66	180.90	56.42	37.09	43.26	69.57
CD40LG (pg/mL)	33	704	4930	10011	15068	16016	9639	5164	7808	11471

A.1.2 Chapter 4 tables

Table A 3: Sensitivity & specificity of the α C1q assay.

α C1q ab Units/mL	Sensitivity % SLE in true positive (TP)	Specificity % non-SLE in true negative (TN)	*PVP + (TP/(TP+FP))%***	**PVN - (TN/(TN+FN))%***
5	89.3	24.2	65.4	54.5
10	69.6	84.8	87.0	62.8
20	57.1	90.9	91.4	55.6
25	50.0	90.9	90.0	50.8
30	41.1	90.9	88.0	46.9
40	33.9	93.9	85.7	44.9
100	16.1	100.0	100.0	41.3

* Predictive positive value = likelihood a positive result indicates presence of disease

** Predictive negative value = likelihood a negative result indicates absence of disease

*** TP = number of values (individuals) above cut-off of interest in the SLE cohort; FP = number of values (individuals) above cut-off of interest in the control cohort; TN = number of values (individuals) below cut-off of interest in the control cohort; FN = number of values (individuals) below cut-off of interest in the SLE cohort.

Table A 4: GraphPad Prism data (detailed) for the above Table A 3.

Cut-off	Sensitivity y%	Sensitivity	95% CI	Specificity %	95% CI	Likelihood ratio	Sensitivity %
> 3.209	98.21	0.9821	90.45% to 99.95%	0	0% to 10.58%	0.9821	98.2
> 3.48	96.43	0.9643	87.69% to 99.56%	0	0% to 10.58%	0.9643	96.4
> 3.793	94.64	0.9464	85.13% to 98.88%	0	0% to 10.58%	0.9464	94.6
> 4.056	94.64	0.9464	85.13% to 98.88%	3.03	0.07669% to 15.76%	0.976	94.6
> 4.336	92.86	0.9286	82.71% to 98.02%	3.03	0.07669% to 15.76%	0.9576	92.9
> 4.551	92.86	0.9286	82.71% to 98.02%	6.061	0.7426% to 20.23%	0.9885	92.9
> 4.763	92.86	0.9286	82.71% to 98.02%	9.091	1.915% to 24.33%	1.021	92.9
> 4.86	91.07	0.9107	80.38% to 97.04%	9.091	1.915% to 24.33%	1.002	91.1
> 4.923	91.07	0.9107	80.38% to 97.04%	12.12	3.403% to 28.2%	1.036	91.1
> 4.967	91.07	0.9107	80.38% to 97.04%	15.15	5.109% to 31.9%	1.073	91.1
> 5.018	91.07	0.9107	80.38% to 97.04%	18.18	6.979% to 35.46%	1.113	91.1
> 5.07	91.07	0.9107	80.38% to 97.04%	21.21	8.98% to 38.91%	1.156	91.1
> 5.132	91.07	0.9107	80.38% to 97.04%	24.24	11.09% to 42.26%	1.202	91.1
> 5.43	89.29	0.8929	78.12% to 95.97%	24.24	11.09% to 42.26%	1.179	89.3
> 5.83	89.29	0.8929	78.12% to 95.97%	27.27	13.3% to 45.52%	1.228	89.3
> 6.003	89.29	0.8929	78.12% to 95.97%	30.3	15.59% to 48.71%	1.281	89.3
> 6.044	89.29	0.8929	78.12% to 95.97%	33.33	17.96% to 51.83%	1.339	89.3
> 6.113	87.5	0.875	75.93% to 94.82%	33.33	17.96% to 51.83%	1.313	87.5
> 6.215	85.71	0.8571	73.78% to 93.62%	33.33	17.96% to 51.83%	1.286	85.7
> 6.411	85.71	0.8571	73.78% to 93.62%	36.36	20.4% to 54.88%	1.347	85.7
> 6.576	83.93	0.8393	71.67% to 92.38%	36.36	20.4% to 54.88%	1.319	83.9
> 6.646	83.93	0.8393	71.67% to 92.38%	39.39	22.91% to 57.86%	1.385	83.9

> 6.689	83.93	0.8393	71.67% to 92.38%	42.42	25.48% to 60.78%	1.458	83.9
> 6.692	82.14	0.8214	69.6% to 91.09%	42.42	25.48% to 60.78%	1.427	82.1
> 6.706	82.14	0.8214	69.6% to 91.09%	45.45	28.11% to 63.65%	1.506	82.1
> 7.009	82.14	0.8214	69.6% to 91.09%	48.48	30.8% to 66.46%	1.595	82.1
> 7.317	82.14	0.8214	69.6% to 91.09%	51.52	33.54% to 69.2%	1.694	82.1
> 7.344	80.36	0.8036	67.57% to 89.77%	51.52	33.54% to 69.2%	1.657	80.4
> 7.388	80.36	0.8036	67.57% to 89.77%	54.55	36.35% to 71.89%	1.768	80.4
> 7.472	80.36	0.8036	67.57% to 89.77%	57.58	39.22% to 74.52%	1.894	80.4
> 7.678	80.36	0.8036	67.57% to 89.77%	60.61	42.14% to 77.09%	2.04	80.4
> 7.907	80.36	0.8036	67.57% to 89.77%	63.64	45.12% to 79.6%	2.21	80.4
> 8.003	80.36	0.8036	67.57% to 89.77%	66.67	48.17% to 82.04%	2.411	80.4
> 8.123	78.57	0.7857	65.56% to 88.41%	66.67	48.17% to 82.04%	2.357	78.6
> 8.276	78.57	0.7857	65.56% to 88.41%	69.7	51.29% to 84.41%	2.593	78.6
> 8.382	78.57	0.7857	65.56% to 88.41%	72.73	54.48% to 86.7%	2.881	78.6
> 8.5	78.57	0.7857	65.56% to 88.41%	75.76	57.74% to 88.91%	3.241	78.6
> 8.573	76.79	0.7679	63.58% to 87.02%	75.76	57.74% to 88.91%	3.167	76.8
> 8.585	75	0.75	61.63% to 85.61%	75.76	57.74% to 88.91%	3.094	75.0
> 8.833	73.21	0.7321	59.7% to 84.17%	75.76	57.74% to 88.91%	3.02	73.2
> 9.173	71.43	0.7143	57.79% to 82.7%	75.76	57.74% to 88.91%	2.946	71.4
> 9.627	71.43	0.7143	57.79% to 82.7%	78.79	61.09% to 91.02%	3.367	71.4
> 10.02	71.43	0.7143	57.79% to 82.7%	81.82	64.54% to 93.02%	3.929	71.4
> 10.07	71.43	0.7143	57.79% to 82.7%	84.85	68.1% to 94.89%	4.714	71.4
> 10.16	69.64	0.6964	55.9% to 81.22%	84.85	68.1% to 94.89%	4.596	69.6
> 10.29	69.64	0.6964	55.9% to 81.22%	87.88	71.8% to 96.6%	5.746	69.6
> 10.73	67.86	0.6786	54.04% to 79.71%	87.88	71.8% to 96.6%	5.598	67.9
> 11.5	66.07	0.6607	52.19% to 78.19%	87.88	71.8% to 96.6%	5.451	66.1
> 12.45	64.29	0.6429	50.36% to 76.64%	87.88	71.8% to 96.6%	5.304	64.3
> 13.59	62.5	0.625	48.55% to 75.08%	87.88	71.8% to 96.6%	5.156	62.5
> 14.98	60.71	0.6071	46.75% to 73.5%	87.88	71.8% to 96.6%	5.009	60.7
> 16.1	58.93	0.5893	44.98% to 71.9%	87.88	71.8% to 96.6%	4.862	58.9
> 18.14	57.14	0.5714	43.22% to 70.29%	87.88	71.8% to 96.6%	4.714	57.1
> 20.18	57.14	0.5714	43.22% to 70.29%	90.91	75.67% to 98.08%	6.286	57.1
> 20.77	55.36	0.5536	41.47% to 68.66%	90.91	75.67% to 98.08%	6.089	55.4
> 21.1	53.57	0.5357	39.74% to 67.01%	90.91	75.67% to 98.08%	5.893	53.6
> 22.44	51.79	0.5179	38.03% to 65.34%	90.91	75.67% to 98.08%	5.696	51.8
> 24.74	50	0.5	36.34% to 63.66%	90.91	75.67% to 98.08%	5.5	50.0
> 25.99	48.21	0.4821	34.66% to 61.97%	90.91	75.67% to 98.08%	5.304	48.2
> 26.68	46.43	0.4643	32.99% to 60.26%	90.91	75.67% to 98.08%	5.107	46.4
> 27.21	44.64	0.4464	31.34% to 58.53%	90.91	75.67% to 98.08%	4.911	44.6
> 27.32	42.86	0.4286	29.71% to 56.78%	90.91	75.67% to 98.08%	4.714	42.9
> 29.9	41.07	0.4107	28.1% to 55.02%	90.91	75.67% to 98.08%	4.518	41.1
> 34.41	39.29	0.3929	26.5% to 53.25%	90.91	75.67% to 98.08%	4.321	39.3
> 36.56	37.5	0.375	24.92% to 51.45%	90.91	75.67% to 98.08%	4.125	37.5

> 36.85	37.5	0.375	24.92% to 51.45%	93.94	79.77% to 99.26%	6.188	37.5
> 37.47	35.71	0.3571	23.36% to 49.64%	93.94	79.77% to 99.26%	5.893	35.7
> 39.76	33.93	0.3393	21.81% to 47.81%	93.94	79.77% to 99.26%	5.598	33.9
> 45.97	32.14	0.3214	20.29% to 45.96%	93.94	79.77% to 99.26%	5.304	32.1
> 52.29	30.36	0.3036	18.78% to 44.1%	93.94	79.77% to 99.26%	5.009	30.4
> 58.75	28.57	0.2857	17.3% to 42.21%	93.94	79.77% to 99.26%	4.714	28.6
> 65.07	28.57	0.2857	17.3% to 42.21%	96.97	84.24% to 99.92%	9.429	28.6
> 67.45	26.79	0.2679	15.83% to 40.3%	96.97	84.24% to 99.92%	8.839	26.8
> 70.87	25	0.25	14.39% to 38.37%	96.97	84.24% to 99.92%	8.25	25.0
> 75.39	23.21	0.2321	12.98% to 36.42%	96.97	84.24% to 99.92%	7.661	23.2
> 80.16	21.43	0.2143	11.59% to 34.44%	96.97	84.24% to 99.92%	7.071	21.4
> 84.07	19.64	0.1964	10.23% to 32.43%	96.97	84.24% to 99.92%	6.482	19.6
> 86.95	17.86	0.1786	8.91% to 30.4%	96.97	84.24% to 99.92%	5.893	17.9
> 99.43	16.07	0.1607	7.622% to 28.33%	96.97	84.24% to 99.92%	5.304	16.1
> 110.4	16.07	0.1607	7.622% to 28.33%	100	89.42% to 100%		16.1
> 111.3	14.29	0.1429	6.375% to 26.22%	100	89.42% to 100%		14.3
> 116	12.5	0.125	5.176% to 24.07%	100	89.42% to 100%		12.5
> 135.1	10.71	0.1071	4.035% to 21.88%	100	89.42% to 100%		10.7
> 160.7	8.929	0.08929	2.963% to 19.62%	100	89.42% to 100%		8.9
> 181.1	7.143	0.07143	1.98% to 17.29%	100	89.42% to 100%		7.1
> 237.5	5.357	0.05357	1.119% to 14.87%	100	89.42% to 100%		5.4
> 290.7	3.571	0.03571	0.4355% to 12.31%	100	89.42% to 100%		3.6
> 334.1	1.786	0.01786	0.0452% to 9.553%	100	89.42% to 100%		1.8

Table A 5: Anti-C1q levels data for SLE patients and controls used to calculate column 4 and 5 in table A 3.

SLE patients	Controls
3.1910	3.8525
3.2260	4.4120
3.7330	4.6890
4.2585	4.8817
4.8365	4.9630
5.1885	4.9700
6.0700	5.0657
6.1560	5.0735
6.5475	5.6700
6.6900	5.9885
7.3340	6.0165
8.0260	6.2737
8.5675	6.6027
8.5770	6.6875
8.5920	6.6935
9.0740	6.7180

10.0910	7.3003
10.3470	7.3540
11.1125	7.4215
11.8820	7.5210
13.0140	7.8335
14.1623	7.9785
15.8010	8.2200
16.3940	8.3315
20.4620	8.4310
21.0725	9.2717
21.1180	9.9820
23.7610	10.0557
25.7150	10.2315
26.2553	19.8890
27.1040	36.8000
27.3095	63.3603
27.3260	109.9157
32.4830	
36.3280	
36.9090	
38.0385	
41.4870	
50.4440	
54.1350	
66.7855	
68.1115	
73.6193	
77.1538	
83.1695	
84.9740	
88.9340	
110.9030	
111.6880	
120.3260	
149.8655	
171.4375	
190.7380	
284.3430	
297.0940	
371.1618	

Table A 6: Data shows the number of individuals above/below cut-offs used for calculation in table A 3.

Cut-off	Above	Below	Above	Below
5.022	51	5	27	6
> 10.02	40	16	6	27
> 20.18	32	24	3	30
> 25.99	27	29	3	30
> 34.41	22	34	3	30
> 45.97	18	38	3	31
> 52.29	17	39	3	31
> 65.07	16	40	3	32
> 70.87	14	42	3	32
> 80.16	12	44	3	32
> 99.43	9	47	3	32
> 110.4	9	47	0	33

A.1.3 Chapter 6 tables

Table A 7: Correlation matrix between DNASE I and VNTR (*HumDN1*) loci and other parameters in SLE patients and healthy controls.

Spearman Correlations	Age	C1Q	C3	C4	DNASE I	α C1q ab	DNASE I Allele	VNTR (HUMDN1) Genotype	Ethnicity	CRP	Ds-DNA AB
Age		-0.115	0.063	0.000	0.034	-0.146	0.240	0.163	-0.308 ($P=0.0211$)	0.014	-0.275 ($P=0.0424$)
C1Q	0.068		0.248	0.120	0.263 ($P=0.0499$)	0.088	-0.155	0.008	-0.009 ($P=0.0244$)	0.325 ($P=0.0244$)	0.241
C3	-0.002	-0.020		0.653 ($P<0.0001$)	0.017	-0.131	0.230	0.304 ($P=0.0227$)	-0.075	0.239	-0.118
C4	-0.068	0.085	0.116		-0.045 ($P=0.0349$)	-0.274 ($P=0.0349$)	0.205	0.279 ($P=0.0376$)	0.184	0.203	-0.087
DNASE I	0.131	0.230	0.225	-0.141		0.038	-0.057	-0.072	-0.094	-0.063	0.059
α C1q ab	-0.565 ($P=0.0006$)	-0.024	0.057	-0.246	-0.368 ($P=0.0349$)		-0.026	-0.073	-0.115	0.136	0.502 ($P=0.0001$)
DNASE I Allele	-0.086	0.017	-0.197	0.254	-0.164	0.183		0.760 ($P=0.0001$)	-0.017	-0.142	-0.188
VNTR (HUMDN1) Genotype	0.042	0.214	-0.020	0.228	0.118	-0.066	0.569 ($P=0.0005$)		-0.137	0.091	0.058
Ethnicity	-0.388 ($P=0.0257$)	0.322	-0.150	-0.008	0.063	-0.062	-0.067	-0.012		-0.173	0.029
CRP											0.406 ($P=0.0047$)
Ds-DNA AB											

The table shows the Spearman correlation coefficients and P values (in brackets where significant) in the SLE cohort (above the diagonal grey line) and in the control cohort (below the grey diagonal line). Shaded grey area in the control group correspond to unavailable data. P values below 0.05 were considered significant.

Table A 8: Contingency table of DNASE I and VNTR (*HumDN1*) loci in SLE patients and healthy controls.

VNTR (<i>HumDN1</i>) & DNASE I genotypes		3/3		3/4		4/4		4/5		4/6		5/5		5/6		6/6	
		CTRL	SLE	CTRL	SLE	CTRL	SLE	CTRL	SLE	CTRL	SLE	CTRL	SLE	CTRL	SLE	CTRL	SLE
1/1	0	0	0	0	2 (3)	3 (3)	3 (3)	1 (9)	3 (3)	0	1 (3)	0	0	0	0	0	0
1/2	0	1 (3)	0	0	0	1 (3)	0	9 (3)	¹⁰ 6 (18) (18)	6 (18)	¹⁰ (18)	1 (3)	3 (3)	1 (3)	2 (3)	0	0
2/2	0	0	0	0	0	0	0	2 (3)	2 (3)	1 (3)	0	2 (3)	3 (3)	4 (12)	12 (21)	2 (3)	4 (3)

Table A 9: Linkage disequilibria for haplotypes comprising the loci DNASE I on the left and VNTR (*HumDN1*) on the right.

Population	Haplotype (DNASE I/VNTR (<i>HumDN1</i>))	Number (%)	p- value (X2)	Population	Haplotype (DNASE I/VNTR (<i>HumDN1</i>))	Number (%)	p-value (X2)
SLE	1/3	3 (2.7)	0.0821 (3.02)	Controls	1/3		Not observed
	1/4	22 (19.6)	0.0001 (32.67)		1/4	15.5 (23.5)	0.0001 (19.03)
	1/5	12 (10.7)	0.0127 (6.21)		1/5	7 (10.6)	0.0652 (3.40)
	1/6	7 (6.2)	0.0002 (13.97)		1/6	3.5 (5.3)	0.0098 (6.67)
	2/3	1 (0.8)	0.0821 (3.02)		2/3		Not observed
	2/4	12 (10.7)	0.0001 (32.67)		2/4	11.5 (17.5)	0.0001 (19.03)
	2/5	29 (25.9)	0.0127 (6.21)		2/5	16 (24.2)	0.0652 (3.40)
	2/6	26 (23.2)	0.0002 (13.97)		2/6	12.5 (18.9)	0.0098 (6.67)

A.2 Figures

A.2.1 Chapter 2 figures

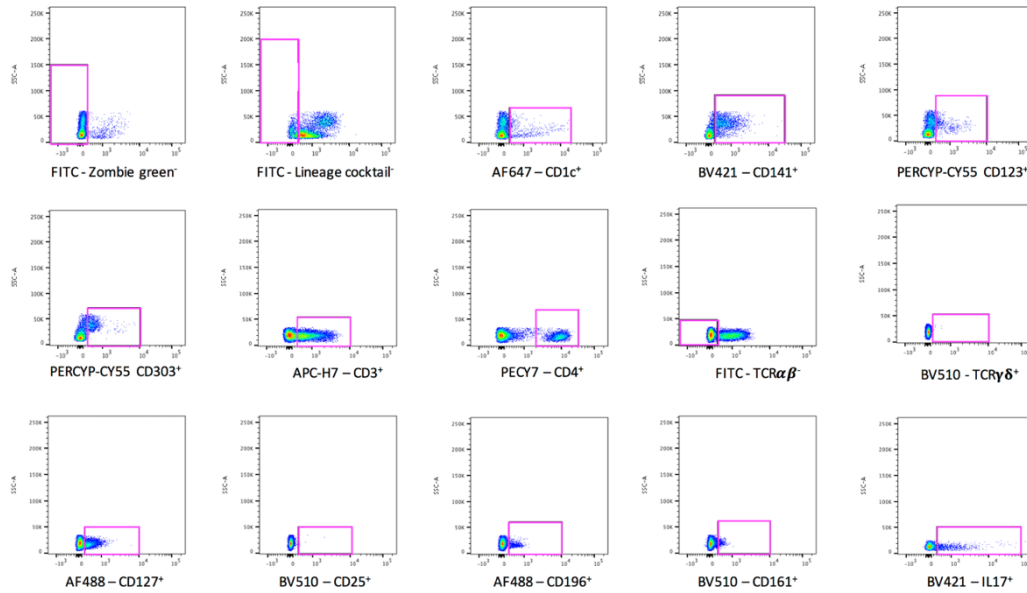


Figure A 7.1: Gates locations based on single stains and isotype controls.

A.2.2 Chapter 3 figures

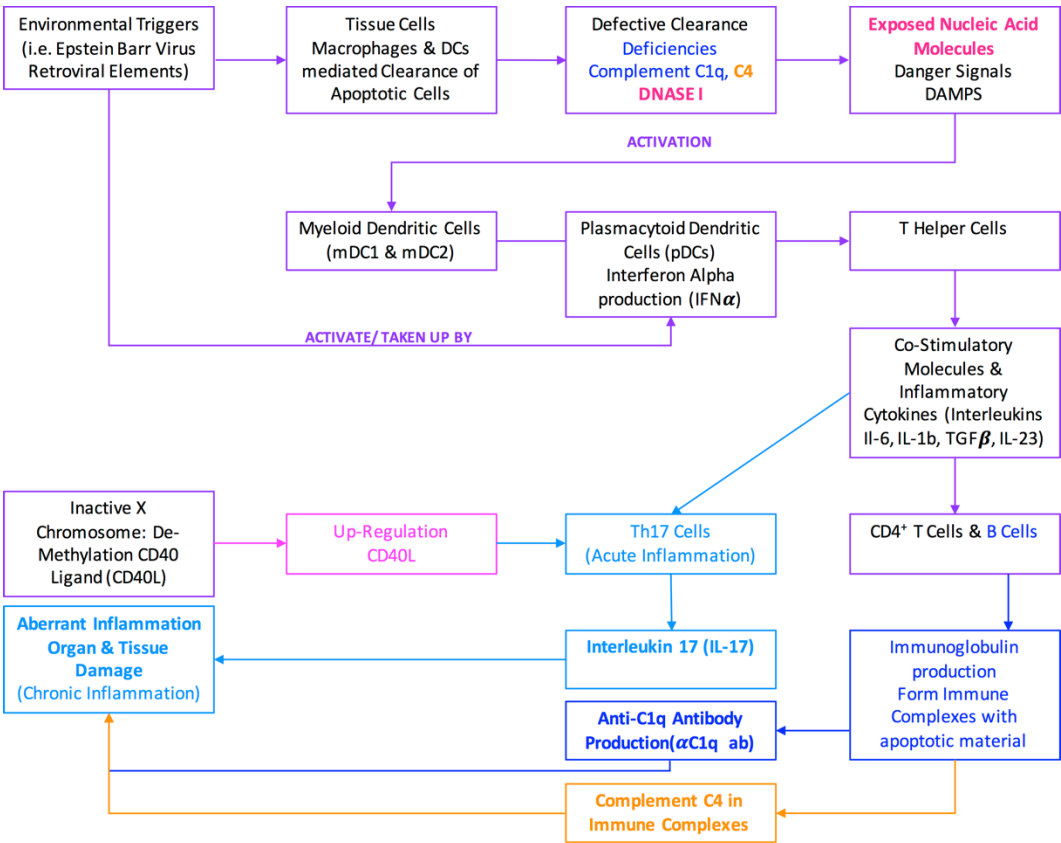


Figure A 7.2: Proposed theoretical cellular mechanism underlying SLE pathology.

A.2.3 Chapter 5 figures

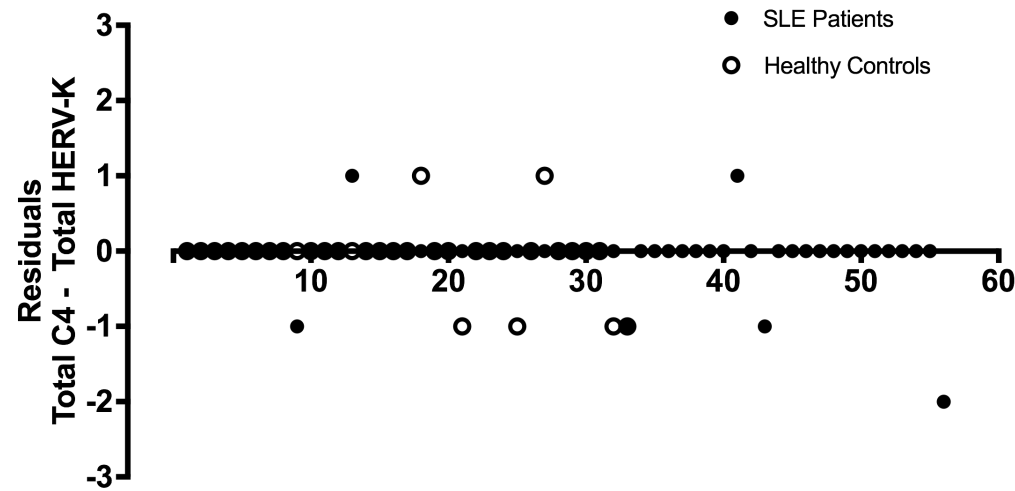


Figure A 7.3: Graphical representation of two assay comparison in ascending order of serum [C4]. Differences between the Taqman™ C4A + C4B assay and the Taqman™ Custom assay for HERV-K retroviral element in C4 genes. Aggregate of SLE patients and controls in ascending order of serum concentrations

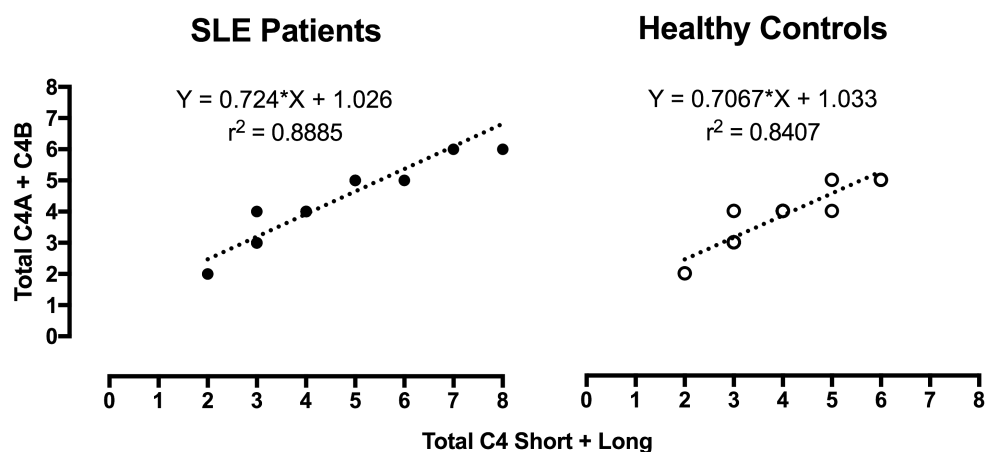


Figure A 7.4: Regression analysis of the C4 and HERV-K gene copy numbers.

Linear regression showing the association between the C4A & C4B GCN and C4 short & long in SLE patients and healthy controls. Linear equation and correlation coefficients are indicated on the graphs.

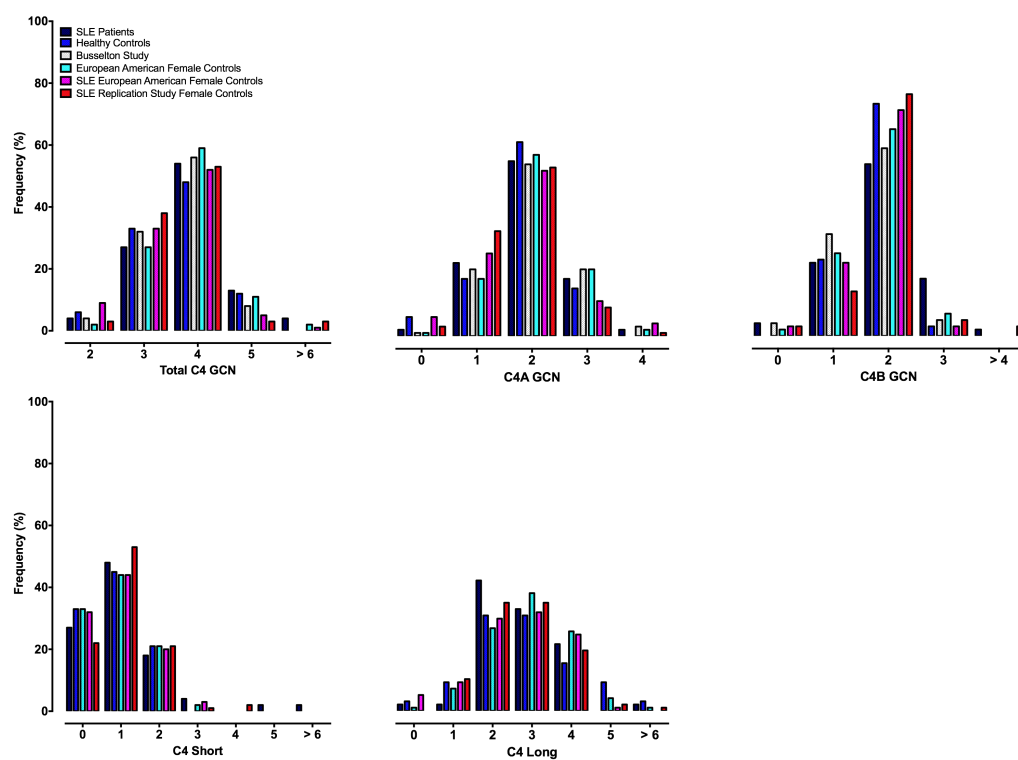


Figure A 7.5: Distribution of gene copy numbers compared to published data.

Data showing total C4, C4A, C4B, and HERV-K present (C4 long) or HERV-K absent (C4 short) genes in SLE and control cohorts compared with previously published data from Yang and colleagues (81).

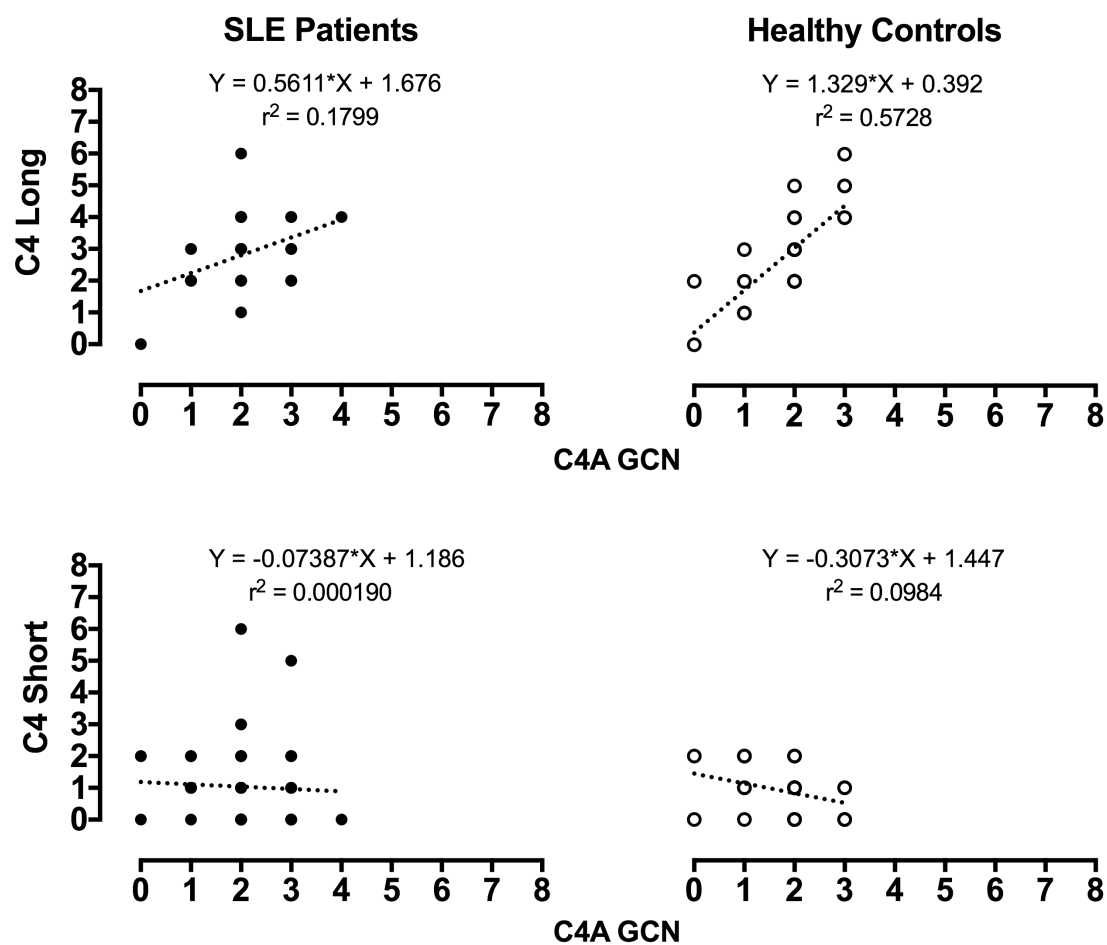


Figure A 7.6: Linear relationship between C4A genes and HERV-K retroviral element.

Linear relationship between C4 short, Long and C4A genes in SLE patients (left hand side graphs) and healthy controls (right hand side graphs). Equations and r^2 values indicated on the graphs.

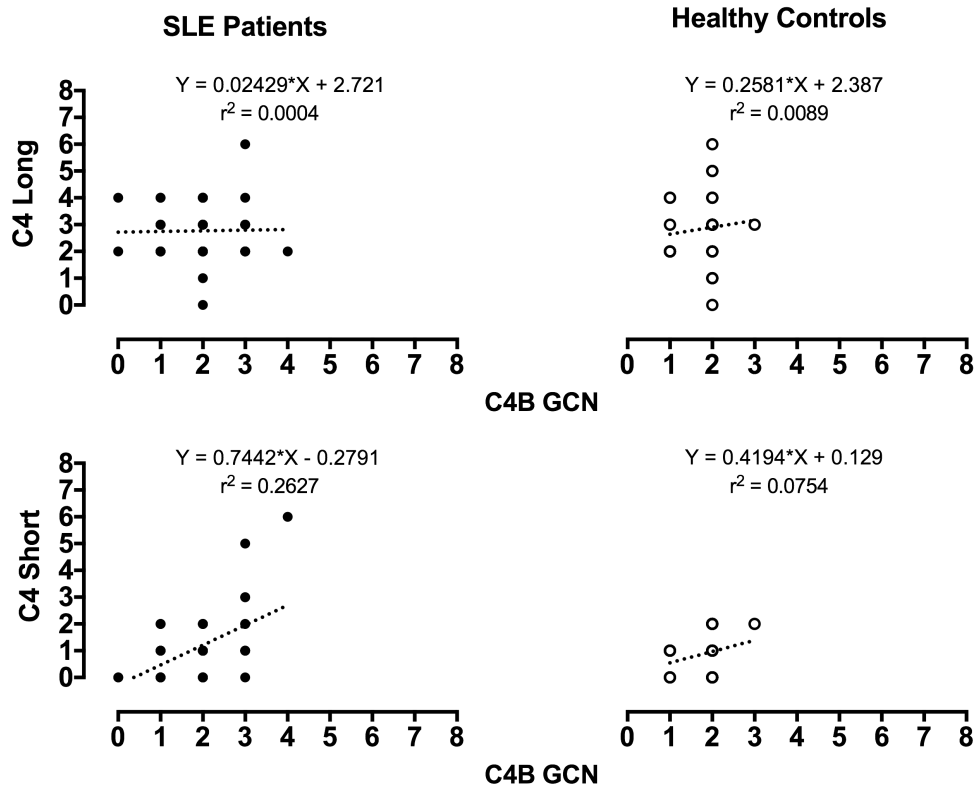


Figure A 7.7: Relationship between HERV-K and C4B genes.

Linear relationship between C4 short, Long and C4B genes in SLE patients (left hand side graphs) and healthy controls (right hand side graphs). Equations and r^2 values indicated on the graphs.

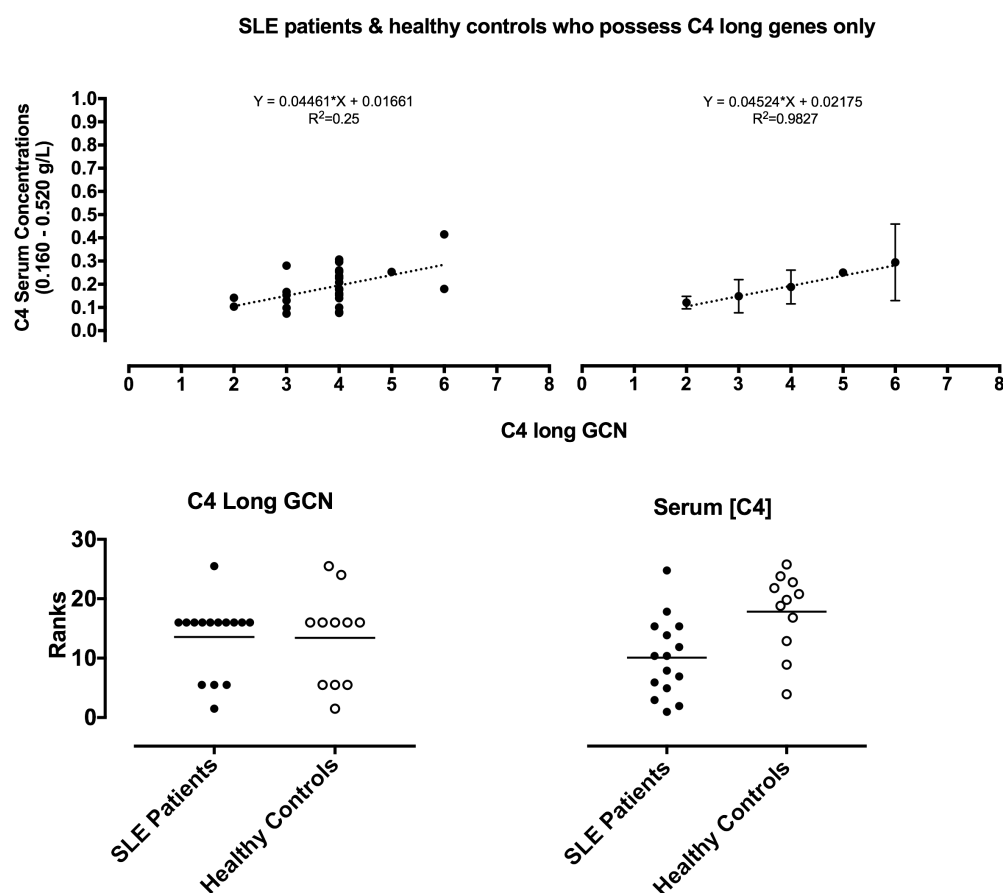


Figure A 7.8: Serum [C4] stratified with HERV-K gene copy number in C4 long genes only.

The top two graphs show a linear regression analysis in SLE patients (left hand side graphs) and healthy controls (right hand side graphs) with C4 long genes only (i.e. they do not have any short C4 genes). Serum C4 concentrations (0.160 – 0.520g/L) = reference ranges in the healthy population. Equation indicated and r^2 values correspond to the analysis performed on individual data points and the mean within the data points respectively. The bottom two graphs show a Mann-Whitney analysis of ranks for the C4 long GCN (left hand side) ($P = 0.9595$ NS) and for the serum [C4] mean concentrations (right hand side) ($P = 0.0087$) between the SLE patients and healthy control.

Appendix B Publications by Margery-Muir et al., used in this thesis

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E-mail address:	Audrey.muir@postgrad.curtin.edu.au
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Our reference:	AUTREV1958
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B.1.2 Anti-C1q Antibodies by Elisa in Systemic Lupus Erythematosus

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Awaiting EIC Decision		View Submission		

B.2 Published articles

B.2.1 Gender Balance in patients with systemic lupus erythematosus (Autoimmunity Reviews)

Autoimmunity Reviews 16 (2017) 258–268



Contents lists available at ScienceDirect

Autoimmunity Reviews

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Review

Gender balance in patients with systemic lupus erythematosus



Audrey A. Margery-Muir^{a,□}, Christine Bundell^b, Delia Nelson^a, David M. Groth^a, John D. Wetherall^a

^a School of Biomedical Sciences, Curtin University, GPO Box U1987, Bentley, Western Australia 6845, Australia

^b Queen Elizabeth II Medical Centre, PathWest, Sir Charles Gairdner Hospital, Hospital Avenue, Perth 6009, Australia

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abstract

Factors are reviewed that contribute to the contemporary view of a disproportionate prevalence and incidence of SLE in females. Recent studies on the epidemiology of SLE report that global incidences and prevalences of SLE for Caucasian and Black populations are of the order of 5.5 and 13.1 per year and 81 and 212 per 100,000 persons respectively. Both parameters displayed age dependent variation over a 90-year lifespan. The female to male (F:M) incidence of SLE varied with age, being approximately 1 during the first decade of life, followed by a sharp increase to 9 during the 4th decade, thence declining in subsequent decades before an increase during the 7th or 8th decade. A cognate review of SLE diagnosis in neonates revealed a F:M ratio of ≈ 1.2 , consistent with the epidemiology review and the sporadic nature of SLE. Notional estimates of disease duration showed a steady increase from a base level for both males and females. The linear trend line for males was always lower than the trend line for females, supporting clinical experience that SLE is a more severe disease in males. Over a 14-year interval ending in 2012, the notional duration of SLE increased from 10–15 years to 20–25 years, probably reflecting advances in diagnosis and clinical practice.

A metastudy of SLE concordance in twins revealed a 75% discordance in monozygotic twins compared to a 95% discordance in dizygotic twins confirming the importance of environmental factors in susceptibility to SLE. The elevated discordance in dizygotic SLE twins (and between siblings) suggests a role for the intrinsic genomic sexual dimorphism due to divergence of Y chromosome regulatory loci from their X chromosome homologues due to lack of recombination of mammalian sex chromosomes over evolutionary time.

Estimates were made of the incidences of SLE in males and females based on population data for nine autosomal deficiency loci of major effect, plus expected male prevalence associated with Klinefelter's syndrome and female prevalence associated with Triple X syndrome. These genetic abnormalities accounted for $\approx 4\%$ of female and $\approx 23\%$ of male Caucasoid prevalence and for SLE resulting in a F:M ratio of ≈ 0.17 . It may be deduced therefore that the impressive preponderance of SLE in females arises from a combination of environmental triggers and susceptibility loci of relatively small effect acting between the interval from the mini-puberty of childhood to the peak of reproductive adulthood. It is in this cohort of females, and especially in the Black population, that combinations of loci of minor effect acting together with environmental factors initiate defective apoptosis resulting in consequential autoimmune disease especially SLE. We postulate that because apoptosis is itself a very complex process, and defective apoptosis is an important contributor to SLE, there will be many combinations of susceptibility loci and environmental stimuli that can result in SLE (and other autoimmune disease(s)), of varying severity.

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* Corresponding author.

E-mail addresses: Audrey.muir@postgrad.curtin.edu.au (A.A. Margery-Muir), chris.bundell@health.wa.gov.au (C. Bundell), delia.nelson@curtin.edu.au (D. Nelson), d.groth@curtin.edu.au (D.M. Groth), j.wetherall@westnet.com.au (J.D. Wetherall).

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1. Introduction

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease characterised by the presence of autoantibodies against chromatin, including dsDNA, and inflammation of various organs, especially the kidney and skin (recent reviews [1,2]). For many years SLE has been considered an archetypal hypersensitivity state mediated by inflammatory processes following organ specific deposition of immune complexes with consequential activation of inflammation pathways including the complement system [2]. The disease is managed by anti-inflammatory and immunosuppressive therapies that significantly reduce mortality rates, but do not effect cures [3].

The aetiology of SLE is unclear, however ineffectual clearance of cellular debris and chromatin during apoptosis has been proposed as the important effector mechanism in many instances and there is considerable evidence to support this hypothesis (reviewed by [4]; and [5,6]). The incidence of SLE is sporadic, however familial disposition is well described and is often associated with known deficiency states. The latter include deficiency states in the early acting components of the classical complement pathway [2] and TREX1 activity [7,8].

The overall observed and widely reported gender imbalance in persons diagnosed with SLE is impressive. As estimated in this report, ≈ 9 females are diagnosed with SLE for every male, although as will be seen this ratio varies between ethnic populations and with age of the test population. It is proposed the extraordinary preponderance of SLE in females of reproductive age provides important clues to the underlying triggers that lead to SLE in susceptible persons. This review will seek to identify those factors that contribute to the disproportionate prevalence SLE in females.

2. Incidence and prevalence of SLE in human populations

There have been many reports of the incidence and prevalence of SLE in diverse human populations. These studies have shown higher prevalences in women of reproductive age (20–40 years) and in women of Black or Asian ancestry (reviewed by [9,10]). Virtually all studies report an impressive female to male (F:M) ratio. Estimates used in this review of incidence and prevalence of SLE for Caucasoid and Black populations are based on the studies of [11,12] because they discriminate clearly between these two ethnic populations. Mean incidences and prevalences of SLE for Caucasian and Black populations are 5.5 and 13.1, and 81 and 212 per 100,000 persons respectively. The F:M ratio is 9 for both ethnic groups. Data are also included of estimates from two very recent European studies of ethnically mixed populations from the United Kingdom (UK) [13] and France [14]. A fifth report [15] includes data for a small population of indigenous Australians. A summary of the prevalence and, incidence rates and F:M ratios from these studies is shown in Table 1. It is in the nature of epidemiological research that results are sensitive to the methods and definitions used. Differences between the four reports summarised in Table 1 reflect variations in database composition and disease definitions as well as the composition of the populations interrogated together with any environmental factors that may be involved. Despite these limitations, a consistent epidemiology of SLE emerges.

Both incidences and prevalences are higher for females than for males, and the increased susceptibility of individuals from African descent for SLE is clearly apparent. It is interesting to note that the higher incidence and prevalence rates of SLE reported by Bossingham, D. [15] for indigenous Australians in North Queensland are similar to those for European and North American individuals from African descent. Given the long isolation of indigenous Australians, this observation very likely reflects their more closely shared African genetic ancestry.

The gender imbalance ratio based on prevalences is ≈ 10 for all studies. The data of Rees and Arnaud [13,14] included variation in both incidence and prevalence rates as a function of age by decade and it seems clear that SLE may be newly diagnosed in all decades of life. The pronounced increase in incidence of SLE in females between 20 and menopause is clearly seen and contrasts with the slower but steady increase in male diagnosed SLE over their lifetimes. These observations translate into highly variable F:M ratios based on incidences (and prevalences) over a life time and probably account for the variation in ratios often reported in the literature. There are two peaks in the F:M ratio based on incidences – these occur in the third decade (20–29 years) due to the increased susceptibility of females of reproductive age and again in persons older than 79 years. A simplistic measure of disease duration (in years) is the ratio of prevalence to incidence. We have used the data of Rees et al. [13,16] to show this graphically in Fig. 1 for both males and females.

Fig. 1 shows that over a human lifetime disease duration is shorter in males than in females which is consistent with the many reports that conclude that SLE is more severe in males and especially in children [17–19]. The steady increase in disease duration over adulthood likely reflects less severe disease profiles and rapid improvements in the diagnosis and treatment of this disease with consequent increased longevity. This point is reinforced by Rees et al. [13] who estimated temporal incidences and prevalences for their mixed UK population over the interval 1999 to 2012. Disease duration estimates for these data are presented in Fig. 2 and show a steady increase in disease longevity.

Table 1
Meta-analysis of incidence and prevalence of SLE per 100,000 person years from 4 seminal studies and 1 Australian study.

Data source	Gender	Caucasians		Black		Overall	
		Incidence	Prevalence	Incidence	Prevalence	Incidence	Prevalence
[12]	Females	6.3	86.7	12.8	186.3	9.3	127.8
	Males	1.2	8.7	2.1	19.3	1.6	12.4
	F:M ratio	5.3	10.0	6.1	9.7	6	10
[11]	Females	4.7	59	13.4	196.2	9.4	131.1
	Males	0.7	7.5	3.2	23.7	1.7	14.9
	F:M ratio	6.7	7.9	4.2	8.3	6	9
[14]	Females					5.51	79.1
	Males					0.92	11.83
	F:M ratio					6.0	6.7
[13]	M & F	6.73	134.53	22.5*	247.62*	8.34	167.62
						1.44	24.82
	F:M ratio					5.8	6.8
[15] (Aust. indigenous)			30.2**		92.8		45.3
	F:M ratio		6.5**		5.5		6.2

*Average from Black African, Caribbean & other. ** Estimated from data included in paper. Grey shaded boxes represent non-available or not applicable data.

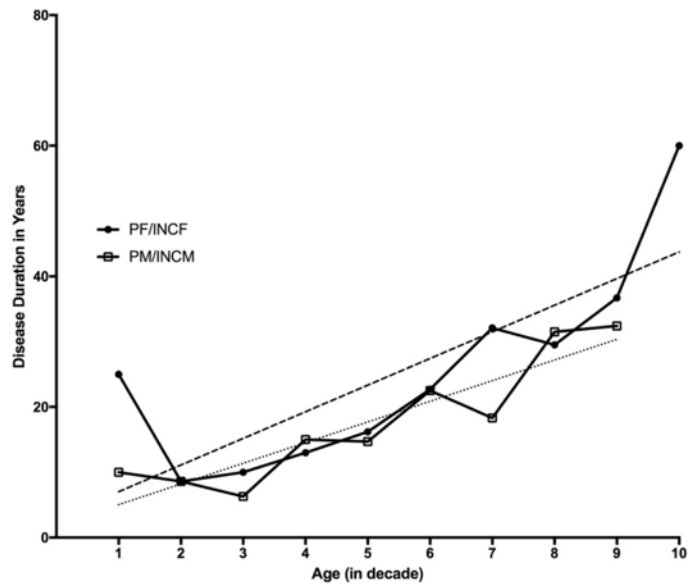


Fig. 1. Duration of SLE per decade estimated from age based prevalence to incidence ratio in years. Estimates derived from the data of Rees [13,16].

Jianguang Ji and colleagues [20] reviewed the gender specific incidence of autoimmune disease in 403,757 subjects registered in the Swedish national disease databases. Their conclusions are generally supportive of the view reported herein that female predominance of

autoimmune diseases may be less striking than previously believed. They also reported that the median age of onset of SLE was 49 years and 58 years for females and males respectively; these values are in close agreement with those of Rees et al. [13] whose data for the year

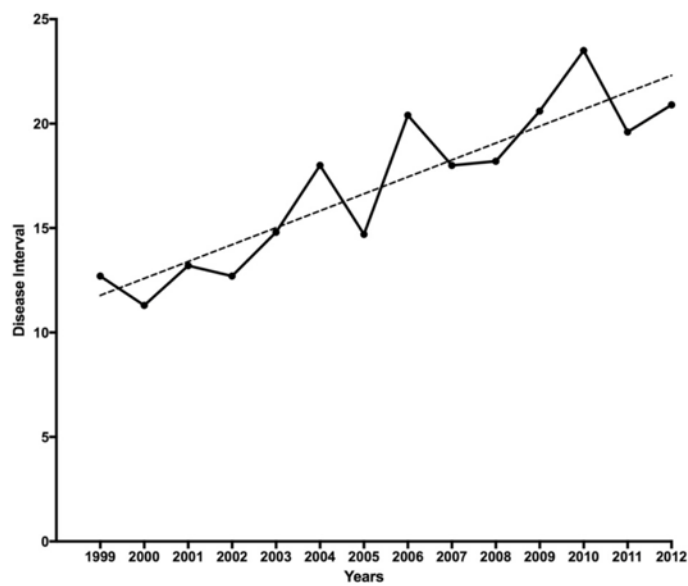


Fig. 2. Estimated duration of SLE in a mixed UK population per calendar year estimated from the data of Rees [13]. Disease duration (years) = prevalence/incidence per year.

2012 implies values of 52 and 62 for females and males respectively in the UK populations. In the latter case the median age of onset of SLE was greater for females than males only for the interval from the 3rd to 6th decades of life.

1. Imbalance of sex ratio in offspring of SLE mothers

A possible explanation for the female preponderance of SLE is loss of male fetuses during the pregnancies of mothers with SLE. This hypothesis was initially addressed by Oleinick [21] who reported a significant decrease in the ratio of live born male siblings to total live born siblings to SLE diagnosed parents. In 1998 Wallace [22] speculated that the reason for the rarity of male lupus could be the presence on the Y chromosome of a "lethal gene" leading to miscarriage of a male fetus, who, if born, would be at a very high risk of developing SLE. Moorthy and colleagues [23] studied a related question by asking whether children with SLE have fewer male siblings than would be expected. They reported that the proportion of male siblings for SLE probands is 0.39, consistent with the families of children with SLE being deficient of male children. The same phenomenon was not observed in the children of probands with pauci-articular onset juvenile rheumatoid arthritis (PaJRA) which also exhibits female preponderance and systemic juvenile onset rheumatoid arthritis (SoJRA) which does not exhibit a female preponderance [23].

Aggarwal et al. [24] determined the sex distribution of 6056 siblings from 2579 women diagnosed with SLE on samples from patients registered with the "The Lupus Family Registry and Depository" [25]. A significant excess of female children relative to male children was observed in this comprehensive study; we estimated the decreased proportion of male siblings in families with an SLE mother to be approximately 13%. A more recent large study has not confirmed the reports described above. Dar and colleagues [26] used the records of the Maccabi Health Services (Israel) to determine the proportion of live born males in 380,472 offspring of women free of rheumatoid arthritis (RA), SLE and psoriatic arthritis (PsA) and in 182,073 women with at least one of these three diseases. Patients with SLE or RA did not differ from the general population by the sex of their offspring. It is difficult to reconcile the differing conclusions from these two important studies. It is possible that the selection of SLE patients in the Israeli Maccabi Health Services database was too sensitive and the inclusion of dubious cases may have obscured a deficiency of male offspring.

In a related study of male only SLE, Aggarwal and colleagues [27] described five families with only male SLE. They observed that white men with SLE were five times more likely to have a child with SLE than were white women with SLE. This likelihood was not observed in Individuals from African descent. Fluorescent *in situ* hybridisation studies in the five families with only male SLE failed to detect an equivalent of the "Y-linked autoimmune accelerating locus" designated *Yaa*. The *Yaa* locus was initially described by Murphy and Roths as a Y chromosome locus that accelerated SLE disease development in male BXSB hybrid mice [28]. It is now known that *Yaa* arises from a translocation of X-linked genes onto the Y chromosome of the BXSB mice leading to a duplication of *Ms131*, *TLR7*, *TLR8*, *Tmsb-4x* and *Rab9* loci [29,30]. As described above, the elevated F:M ratio for SLE only occurs during the reproductive stages of a woman's life and the gender ratio of neonates and children diagnosed with SLE is much closer to unity. These observations are inconsistent with preferential male fetal loss in SLE.

2. Genetic factors affecting the gender ratio of SLE in humans

2.1. Twin studies

Many reports have demonstrated a familial nature of SLE. However twin studies have shown only 25–30% concordance between identical twins and little or no concordance between fraternal twins. Interestingly, our review of 18 case reports and studies of twins with SLE between 1975 and 2013 revealed a remarkable discordance rate. Indeed, these

reports indicate total discordance rates in monozygotic and dizygotic twins of 69% and 96% respectively. In calculating the overall rates for monozygotic and dizygotic twin sets, we find that 75% are discordant for SLE and 25% are concordant only. The summary of individual data and references for each study are shown in Table 2. Discordance between monozygotic twins is usually attributed to variation in environmental factors either *in utero* or within the interval between neonatal life and age of diagnosis of SLE. Incomplete information and variation between studies prevented reliable estimates of the F:M ratio of the twin pairs reported in Table 2.

Only a few of the twin studies in Table 2 reported similarity of symptoms, outcomes and times of diagnosis/onset [31–33]. In most instances, there was considerable variation in pathology, serology, and time of onset [34–36]. Indeed, Kuroda and colleagues [34] reported a set of twins who still lived together at time of onset of disease; despite MHC class I and II similarity the diagnosis of SLE for each twin differed by 3 years with only one twin manifesting a lupus psychosis. In a discordant case, both twin sisters presented with *myasthenia gravis* and both received a thymectomy 14 years apart; the twins were diagnosed 2 years apart and followed for 28 years. The concordant twins reviewed by Del Boz and colleagues [32] were males with Klinefelter's XXY karyotype, similar pathologies and age of diagnosis.

Fraga and colleagues [37] studied epigenetic profiles in monozygotic twins and observed distinct epigenetic patterns in each twin. They showed that these differences were associated with age, the time spent together and their medical history. Moreover, gene expression profiles showed a four-fold differential expression in older twins and a higher number of overexpressed genes and hypo-methylation that may lead to increased transcription and activation of these genes. It seems clear that although twins are viewed as genetically identical, distinct epigenetic signatures reflecting environmental differences may generate distinct phenotypes that could account for discordance in autoimmune diseases, including SLE.

2.2. Neonatal SLE

Nineteen reports of neonatal SLE between 2013 and 2016 were reviewed and are summarised in Table 3. In 114 neonates with SLE there were 63 females and 51 males to give a F:M ratio of 1.2. This result is consistent with the data presented in Fig. 1 showing the incidence of SLE in male and female children in the first decade of life. Of the 114 neonates with SLE the mothers of 46 were not recorded as having tests or symptoms of autoimmune disease, especially SLE. This result is consistent with sporadic SLE being frequent.

3. SLE associated with chromosomal mutations

The increased incidence of SLE in females relative to males suggests an important role for the X chromosome in the predisposition to SLE. Nondisjunction of the X chromosome during meiosis gives rise to males with an XXY karyotype and subsequent abnormalities referred to as Klinefelter's syndrome (KS). Although reported cases are low (about 30 cases between 1970 and 2009), the prevalence of SLE in males with KS is increased ≈ 14 times relative to normal males [38]. The disease phenotype in KS males is similar to that in females. In a follow-up study to that of Scofield et al. [38], Dillon and colleagues [39] reported that 7 of 286 men with SLE from the Lupus Family Registry and Depository [25] manifested abnormal karyotypes that included 47XXY. In contrast to non-KS men with SLE the disease symptoms of the KS men with SLE were less severe and did not include nephritis. Further, it is clear that not all KS males develop SLE. Men with KS who develop SLE behave as a cohort more like women with mild SLE. The prevalence of KS in Australia is approximately 1115 per 500,000 males [40] with most KS patients being initially identified as adults presenting with infertility.

Table 2
SLE concordance/discordance in human twin [92–104].

Study	Gender	Monozygotic discordance	Dizygotic discordance	Monozygotic concordance	Dizygotic concordance	Total monozygotic sets	Total dizygotic sets
[92]	F	1	n/a	0	n/a	1	n/a
[31]	All F, 1 M	6	0	13	1	19	1 14
[93]	F	3	3	3	0	6	3
[94]	M	1	n/a	0	n/a	1	n/a
[95]	F	1	n/a	0	n/a	1	n/a
[96]	M	1	n/a	0	n/a	1	n/a
[35]	F	1	n/a	0	n/a	1	n/a
[97]	1 MZ M	8	10	1	0	9	10
[98]	F & 1 DZ M co-twin	3	6	1	0	4	6
[99]	F, 1M	9	n/a	0	n/a	9	n/a
[100]	F	4	n/a	1	n/a	5	n/a
[34]	F	0	n/a	1	n/a	1	n/a
[101]	F	1	n/a	0	n/a	1	n/a
[32]	M	0	n/a	1	n/a	1	n/a
[36]	F	1	n/a	0	n/a	1	n/a
[102]	F, 1 DZ M	6	4	0	0	6	4
[103]	F	1	n/a	0	n/a	1	n/a
[104]	F	1	n/a	0	n/a	1	n/a
[33]	F	0	n/a	1	n/a	1	n/a
Total		48	23	22	1	70	24
%		68.6%	95.8%	31.4%	4.2%	74.5%	25.5%
Discordance total		75.5%					
Concordance total				24.5%			

This table shows data from 19 studies published between 1975 and 2013 that included twin sets, monozygotic or dizygotic in which either one or both the siblings had SLE. Grey shaded boxes represent non-available or not applicable data. The gender column shows the gender of the probands: F –females, M –males, MZ –monozygotic, DZ–dizygotic. If the gender status was not clear in the original studies, it was assumed that female twin sets were included.

Harris and colleagues extended these findings by reporting that the KS karyotype was more frequent in males diagnosed with Sjogren's syndrome than in either control populations or rheumatoid arthritis patients [41]; they also confirmed excess KS in men diagnosed with SLE. In a cognate study Liu et al. [42] reported an increased prevalence of SLE and Sjogren's syndrome in a cohort of females with another chromosomal mutation – the 47XXX karyotype, providing further evidence for dosage effects reflecting X chromosome copy number. The prevalence of 47XXX in patients (females) with SLE was estimated to be 0.25%. The prevalence of SLE in females with Turner's syndrome (characterised by the 45XO karyotype) seems to be diminished relative to normal females. Only three cases of SLE in women with the 45XO karyotype have been reported possibly because karyotyping is not usually

performed in these women [43–45], although the Ruas study also described neonatal SLE in a 45XO female infant.

1. Genomic sexual dimorphism - influence of the Y chromosome

Recent sequencing and phylogenetic analysis of the human Y chromosome has provided important new information on intrinsic differences between male and female cells [46,47]. It is now understood that the Y chromosome includes a complement of regulatory genes (UTX/UTY, EIF1AX/EIF1AY, ZFX/ZFY, RPS4X/RPS4Y1, KDM5C/KDM5D, DDX3X/DDX3Y, USP9X/USP9Y and TBL1X/TBL1Y) that have diverged from their X chromosome homologues due to lack of recombination of mammalian sex chromosomes over millions of years. The homologues

Table 3
Summary of neonatal births with SLE from 2013 to 2016 [105–122].

Study	Neonate gender	Neonate ethnicity	Maternal history
[105]	7 males & 8 females	8 Caucasians, 4 individuals from African descent, 2 Asians, 1 unknown Caucasian	Connective tissue disorder in 1 mother
[106]	Female		
[107]	Male	Asian	Sjögren syndrome
[108]	Male	Caucasian (?)	
[109]	Female		
[110]	Male	Arab	Active rheumatoid arthritis
[111]	Female	Asian	
[112]	Male	Caucasian	Antibodies positive*
[113]	Female	SLE	
	Male	Black	None
	Female		Juvenile idiopathic arthritis
	Female		None
[114]	5 Females & 7 males		
[115]	2 Males, brothers (diagnosed 2 years apart)		Antibodies positive*
[116]	Male	Senegalese	
[117]	Male		
[118]	Female		
[119]	34 females out of 58		History systemic sclerosis, SLE or both in some
[19]	6 females 1 male (with inherited complement deficiency)	Arabs	
[120]	Female	Asian American	
[121]	Male	Caucasian	
	Female	South American	
	Female	Caucasian	
	Male	Caucasian	Sjögren syndrome
[122]	Male		Sjögren syndrome

Antibodies positive* indicates that the mother was recorded with positive levels of anti RO/SM antibodies. This table shows data extracted from 19 recent studies reporting on cases of neonatal lupus. Studies included the proband's gender, their ethnicity and their mother's medical history at the time the neonate was diagnosed. Shaded boxed indicate cases where no data was available.

of these regulatory genes on the X chromosome also remain functional on the inactivated X chromosome in females so that they are present in both sexes in two copies, albeit that the Y chromosome variants have diverged significantly from their X chromosome counterparts. Notwithstanding the powerful differential influence of sex hormones on male and female tissues, differences between the divergent regulatory homologues shared by the sex chromosomes create a sexual dimorphism manifested as X and Y-encoded protein isoforms in diverse human tissues. David Page (see [46]) has speculated "whether this dimorphism has a role in diseases, outside the reproductive tract, that occur with greater frequency or severity in males or females". These studies do not explain however the

frequent discordance of monozygotic twins with SLE, including those also manifesting Klinefelter's syndrome (see discussion above). Genomic sexual dimorphism may be further complicated by non-random inactivation of the X chromosome in a female cell – a phenomenon called skewed X chromosome inactivation. Kast was the first to postulate a role for X-inactivation chimerism in female-prevalent autoimmune disease [48]. Jeffrey Stewart extended this hypothesis to propose that the discordance rate of SLE between monozygotic twins may be explained by such twins manifesting different X-inactivation patterns, possibly in a tissue specific manner [49]. There seems to be no published evidence to support a role for skewed X inactivation in SLE.

1. Mitochondrial DNA and SLE

The intrinsic pathway of apoptosis is initiated by intracellular signals resulting in permeabilisation of mitochondria with release of cytochrome *c* into the cytoplasm and subsequent apoptosome activation of the caspase cascade. Perl [50] has recently reviewed evidence confirming increased production of reactive oxygen species (ROS) and consequent oxidative stress in peripheral blood lymphocytes (PBL) from patients with SLE; mitochondria in T cells from patients with SLE exhibit dysfunction characterised by elevated mitochondrial transmembrane potentials. Hence, it is possible that variation in maternally transmitted mitochondrial DNA may predispose to SLE and other inflammatory autoimmune disorders. If genetic variation in mitochondrial DNA is a candidate locus for predisposition to SLE, it is unknown whether there are gender specific differences.

2. Inherited autosomal deficiency states predisposing to SLE

There have been many reports of SLE in kindred groups manifesting an autosomal deficiency state. In these families, the susceptibility to SLE usually reflects a strong association with a specific genetic locus (see Table 4). The best described and longest known autosomal loci of major effect in predisposition to SLE encode complement proteins C1q, C1r, C1s, C2, C4A and C4B. Of these, genes encoding C2 and both C4A and C4B (as well as Factor B) occur within the central region of the human MHC on chromosome 6p21.3 (reviewed by [2]). It is notable

that while these associations between the complement component (complete) deficiency state and SLE are generally strong they are not absolute. The percentage of persons with a complete deficiency state that are concordant for SLE varies from a low of 57% for C2 through to over 90% for C1q (see Table 4).

C1q and C1r deficiencies are very rare and associated with early childhood onset of SLE. Stegert [51] and Van Schaarenburg [52] reported the median age of SLE onset was five and nine years old respectively. Van Schaarenburg [52] showed early age of death in their C1q deficient SLE patients (F:M ratio of $\approx 1.3:1$) with a mortality of 20% at time of follow-up. The C2Q0 allele has been estimated to have a frequency of 0.02 in Caucasian populations resulting in 0.04% of persons with homozygous C2 deficiency states [53–55]. Of these only 10% manifest SLE with a sibling concordance rate of approximately 50% and an F:M ratio of ≈ 7 , the latter figure being closer to the overall ratio for all SLE patients (see Table 1).

Humans have two forms of the complement C4 gene designated *C4A* and *C4B*. Both loci encode isotypic forms of C4 protein (C4A and C4B) each with distinct biochemical properties and each displaying multiple electrophoretic variants reflecting allelic polymorphisms [56,57]. Null alleles designated *C4AQ0* and *C4BQ0* exist at both loci as a result of several well-defined mutations [58–60]. A further level of complexity arises from the propensity of this chromosomal segment to duplicate resulting in as many as four distinct replicons per chromosome each containing either a *C4A* locus or a *C4B* locus [56,57]. Males and females share this complexity and the distributions of serum levels for both C4 proteins are similar in

Table 4
Counting expected SLE cases in a Caucasian population of one million persons (M:F = 1) [123–125].

Deficiency genotype)	Chromosome	Prevalence $\times 10^{-6}$	Concordant with SLE %	Sibling SLE concordance %	Female SLE	Male SLE	F:M ratio concordant siblings**	References
Population	Caucasoid	811	N/A	N/A	730	81	9	This review
	Black	2125			1910	215	9	
C1q	1p36	1	93	90	0.5	0.5	1.3 \approx 1	[123]
C1r and C1s	12p13	1	57	67	0.5	0.5	1.7	[123]
C2	6p21	400 or 100 G	10	58	8.75	1.25	7:1	[123]
Total C4 (C4A + C4B)	6p21	1	75	80	0.7	0.3	2.1 \approx 2	[123]
FcGR	1q23	10	100	N/A	5	5	1	[65, 66]
Complex TREX1#	3p21.31	20 check	100	2% check	10	10	1	[67, 69]
(DNase III exonuclease)								
Extracellular DNASE I & II	$\approx 3p14.3$	1	75		0.5	0.5	1	[72, 124]
Klinefelter's syndrome	Karyotype XXY	1700 [^]	2.5	N/A	0	1.0	N/A	[38, 125]
Triple X syndrome	Karyotype XXX	1000 [^]	4	N/A	4	0	N/A	[45]
No. predicted					30	19		1.6
% predicted					4%	23%		0.17
					1.6%	10%		0.16

*For rare deficiency states with no reliable published incidence/prevalence data, the prevalence has been assumed to be of the order of 1×10^{-6} . # Includes complete deficiency and heterozygous deficiencies. [^] Prevalence per 500K females or males only. Grey shaded boxes and N/A represent non-available or not applicable data.

males and females [61]. Reliable population data for the frequencies of *C4AQ0* and *C4BQ0* null alleles is limited, although in Caucasians the frequencies of the *C4AQ0* and *C4BQ0* alleles are reported as 1% and 3% respectively [2]. Complete C4 protein deficiency is therefore very rare.

Other loci predisposing to SLE are the inhibitory Fc gamma binding receptor (FcGR3B), endogenous DNase (Trex1) and exogenous DNases I and II. Fc receptors link humoral and cellular immunity by facilitating phagocytosis and antibody dependent cell mediated cytotoxicity and are important in modulating the immune response [62]. The human *FcGR* locus on chromosome 1q23 is subject to copy number variation (CNV) [63–65]. This genomic region contains the *FcGR3B* locus and paralogous genes *FcGR2A*, *FcGR3A*, *FcGR2C*, and *FcGR2B*, encoding low-affinity Fc gamma receptors [65]. The inhibitory receptor gene, *FcGR2B*, exhibits a polymorphism within the trans-membranedomain that is associated with SLE [66].

TREX1 (DNase III) is the major 3'–5' DNA exonuclease within mammalian cells. *TREX1* is located on chromosome 3p21.31 and consists of a single exon encoding a homodimeric 314 amino acid polypeptide chain [67]. *TREX1* is an important component of the SET protein, a multitasking protein complex that associates with the endoplasmic reticulum and translocates to the nucleus in response to oxidative stress. In association with SET, *TREX1* causes single-stranded DNA damage during caspase-independent apoptosis activated by granzyme A [67]. Crow and colleagues have described an IFN-driven inflammatory disease associated with *TREX1* loss in human patients (Aicardi-Goutieres syndrome) that is characterised in part by an autoimmune syndrome similar to SLE [67,68]. Lee-Kirsch et al. [69] reported that 9 of 417 SLE patients from the UK, Finland and Germany (males and females not specified) manifested *TREX1* mutations with no mutations in their control cohort. More recently Ellyard and colleagues described a four-year-old female with cerebral SLE caused by a rare homozygous mutation in *TREX1* [70]. Namjou and colleagues reported that the estimated frequency of *TREX1* mutations in their mixed ethnicity SLE cohort was 0.5% [71]. These findings are consistent with mutant forms of *TREX1* having impaired exonuclease activity and therefore predisposing to inflammatory diseases by reduced degradation of dsDNA during apoptosis. Another recent study [72] provides important insights into the digestion of DNA containing microparticles (formed from reverse transcribed retroelements) and its role in preventing autoimmune disease. DNA from apoptotic cells is degraded by the intracellular enzyme DNASE2, whose deletion in mice causes IFN-driven auto-inflammation [73]. These rare but highly penetrant causative mutations are not detected in genome wide studies which may explain part of the missing heritability of SLE as well as provide insights into disease pathogenesis. We

are unaware of any gender bias in the expression of *DNases*.

It is also of considerable interest that endogenous DNase activity is present in blood from healthy persons. DNase activity is increased in the serum of SLE patients experiencing a relapse of disease, but not to the levels seen in healthy controls [74]. Barcelos-Barra and colleagues showed that DNase activity in blood plasma is inhibited by the anti-coagulant EDTA which more closely mimics the physiological state of circulating blood [75]. The genetic basis of DNase activity in human blood has not been described.

Crow et al. [76] have suggested that SLE is a heterogeneous collection of individually rare genetically distinct disorders sharing a common inflammatory pathology. These deficiency states, although relatively rare, go some distance in accounting for the genetic complexity of SLE predisposition (see also Table 4). Further, these autosomal deficiency states are expressed in both sexes and, as expected, do not contribute to the high female prevalence in SLE patients.

1. Estimated prevalence of SLE in males and females due to autosomal loci of major effect and Klinefelter's syndrome

The prevalence of SLE in males and females due to chromosomal mutations of the sex chromosomes and known autosomal loci of

major effect is estimated as shown in Table 4. This table records prevalences of SLE in a Caucasoid population of one million persons (males = females). There is a paucity of data for the prevalences of most of the autosomal deficiency states that are strongly associated with SLE, although it is clear that these are mostly rare deficiency states. To overcome this problem, we have therefore assumed population prevalences for complete C1q, C1r, C1s, C4A + C4B, and *TREX1* (DNase III) deficiency states as 1 in one million, based on inferences from the literature and consider these conservative. Other prevalences are derived from the references shown in Table 4.

As shown in Table 4, these genetic predispositions of major effect account for a greater proportion of male SLE diagnoses than female. Further, based on the differing age dependent incidence and prevalence profiles for SLE most of these male diagnoses occur within the first two decades of life, or later in life. The impressive female preponderance of SLE prevalence arises therefore from the dramatic increase in females diagnosed with SLE in the 3rd and 4th decades coinciding with the reproductive years. If preconditioning *in utero*, or neonatal hormonal status, increases susceptibility to SLE in this cohort, it is in these females that the underlying mechanisms and environmental triggers should be sought. A number of possibilities have been recognized.

2. Microbiome development, immune responsiveness and sex hormones in predisposition to SLE

2.1. Gender differences in early microbiome development and immune responsiveness and predisposition to SLE

Sex based differences affecting both the innate and adaptive immune responses in humans are well known (reviewed by [77]). It is believed that these contribute to differences in the pathogenesis of infectious diseases and responses to viral vaccines in males and females, and the increased susceptibility in females of autoimmune diseases especially during their reproductive years [77,78]. Of interest in this review is the study of Gaudreau and colleagues [79] who reported increased mucosal immune responsiveness of female (SWR × NZB) F1 (SNF1) mice compared with their male counterparts; female SNF1 mice are especially susceptible to SLE like symptoms including proteinuria [80]. Female SNF1 mice not only showed profoundly higher CD154, immune cell densities, but also carried large numbers of interleukin (IL)-17-, IL-22- and IL-9-producing cells in the lamina propria compared to their male counterparts. The intestinal mucosa of female SNF1 mice expressed higher levels of a large array of pro-inflammatory molecules, including type 1 interferons and TLR 7 and 8 even before puberty. This potential link between the immune response of the gut mucosa and SLE suggests that gender specific responses to environmental or dietary antigens may contribute to the gender imbalance of SLE incidence in humans. A further intriguing link between the mucosal immunity and SLE comes from the report by Markle and colleagues that early-life microbial exposures influence sex hormone levels and modify progression to autoimmunity in the non-obese diabetic (NOD) mouse model of type 1 diabetes in an androgen receptor dependent mechanism [81]. The role of microbiota colonization in early life in modulating immune responsiveness has been reviewed by Gensollen et al. [82].

There is a clear role for hormonal factors and environmental interactions in the manifestation of this sexual dimorphism but possible molecular mechanisms are poorly understood. Two recent reports on regulation of immune responses by sex steroids have shown independently that expression of the autoimmune regulator locus (*AIRE*) is decreased in the female thymus relative to the male thymus especially during pre-puberty and the interval known as the mini-puberty of infancy [83,84]. Dragin and colleagues associated diminished *AIRE* expression with estrogen-induced gene methylation, while Zhu and colleagues followed the finding of increased male *AIRE* expression by demonstrating that the androgen receptor binds the *AIRE* promoter and increases *AIRE* expression (both mRNA and protein). In both studies, *AIRE*

expression affected thymic expression of tissue-specific antigens. Finally, both groups showed that susceptibility to autoimmunity disease (in murine models of multiple sclerosis and thyroiditis) depended on sex steroid production in an AIRE-dependent manner. These ground-breaking studies provide renewed interest in the role of *in utero* sex steroid exposure as a predisposing factor to SLE.

The view that sex hormones contribute indirectly to SLE predisposition is supported by rapid increase described previously in the incidence (and prevalence) of SLE in post-pubertal women relative to both prepubertal males and females. It is known that androgen levels in female SLE patients are lower than in control groups regardless of treatment regimen [85].

1.1. Evidence for an elevated *in utero* androgen receptor environment in SLE predisposition

In humans, the ratio of 2nd to 4th digit length (2D4D) is sexually dimorphic with the mean 2D4D being longer in females than males. Over a decade ago Lutchmaya and colleagues [86] showed that the 2D4D ratio on the right hand exhibited a significant negative association between fetal testosterone to fetal estrogen concentration ratios (FT/FE) in amniotic fluid that was independent of sex. More recently Manning and colleagues [89] measured 2D4D ratios in 100 patients with SLE each fulfilling the American College of Rheumatology criteria and 200 controls without SLE (each cohort contained equal numbers of males and females). Their study showed that patients with SLE had decreased 2D4D ratios relative to controls thereby providing indirect evidence that the SLE patients experienced high prenatal testosterone and low prenatal estrogen. Interestingly, men with KS, in which the prevalence of KS is similar to that observed in women, had 2D4D ratios similar to normal women and is consistent with the observations reported above that SLE in KS males is often less severe than in non-KS males and more closely resembles SLE in women of reproductive age [90]. A study of men with and without multiple sclerosis also showed a decreased 2D4D ratio in men with the disease and concluded that low androgen exposure during the pregnancy could be a risk factor for multiple sclerosis [91]. There is a large number of sexually dimorphic traits that show an association with the 2D4D ratio (see for example <http://www.handresearch.com/news/digit-ratio-finger-length.htm>). It seems that the lower 2D4D ratio seen in female SLE patients offers support for elevated androgens during pregnancy being a risk factor for SLE that may act synergistically with physiological synthesis of sex hormones during prepubescent development, especially in females.

2. Conclusions and discussion

As described herein, recent epidemiological data shows that SLE may be first diagnosed in neonates and at all ages until old age. In contrast to males, the incidence and prevalence of SLE increase dramatically in post pubertal females and remain high during the reproductive years reaching peaks between the third and fifth decades. The F:M ratio for incidence, and hence also prevalence of SLE, varies markedly with chronological age and ranges from ≈ 1 to 10 in figures used in this paper. The often quoted value of a F:M ratio of ten applies only over the third to fifth decades of life. This report shows that for children under ten years the F:M ratio is ≈ 1.2 and for elderly adults gradually decreases to ≈ 3 . The latter figure is probably influenced by the increased longevity of females relative to males and the more severe disease often seen in males. It is noteworthy that age dependent prevalence of SLE divided by the age dependent incidence of the disease shows a steady increase over ten decades for both females and males. This ratio is a notional measure of the average duration of disease and is always higher in

females than males. This observation supports clinical reports that SLE is a more severe disease in males than females with shorter survival times.

The markedly increased F:M ratio during the third to fifth decades in women strongly supports a sex hormone mediated predisposition to the incidence of SLE. A similar observation in males with KS, together with a more female like profile of SLE reported in KS males, strengthens this conclusion. An explanation for this observation is likely to be found in the sex hormone modulation of genes on the X chromosome that modify epigenetic profiles in susceptible women. Two observations demand explanation in understanding the genetic predisposition to SLE. Firstly, there is little concordance between monozygotic twins, and secondly the F:M ratio of neonates with SLE is approximately 1. These observations imply that *in utero* environments affecting both sexes equally predispose to the development of SLE. The obvious candidate is temporal differences in the *in utero* sex hormonal environment during pregnancy. That this environmental factor is relevant is supported by both abnormal androgen levels during early pregnancy of mothers with SLE and especially by the decreased 2D4D ratios reported in both males and females with SLE. Given the consistent variation of 2D4D ratios in persons with many types of traits that exhibit gender imbalance, temporal *in utero* androgen imbalance seems to be an important feature of SLE. The dramatic increase in SLE incidence during the reproductive years in women may reflect a synergistic effect of increased estrogen levels in females predisposed during pregnancy to androgen imbalance.

There is much evidence to support the hypothesis that SLE arises from defects in apoptosis - the complex physiological mechanism whereby the detritus of dead and damaged cells is removed from the body following programmed cell death [5,6]. Relatively rare protein deficiency states mediated by autosomal single locus mutant genes are closely associated with SLE as described above. These loci occur in equal frequency in males and females and SLE is often inherited in kindreds in Mendelian fashion. They are examples of rare genes of major predisposition for SLE. The evidence to date is that in a minority of cases the rare deficiency state occurs in persons who not develop SLE as children but may do so later in life. A conservative estimate of the proportion of SLE cases that may be accounted for by this type of deficiency state is $\approx 5\%$ of children within the first decade of life.

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B.2.2 Anti-C1q Antibodies Concentrations by Elisa in Systemic Lupus erythematosus (Lupus: Open access)



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Research Article

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Anti-C1Q Antibodies Concentrations by Elisa in Systemic Lupus Erythematosus

Audrey A Margery-Muir^{1*}, John D Wetherall¹, David M Groth¹ and Christine Bundell²

¹School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, GPO box U1987 Bentley Western, Australia

²PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Hospital Avenue, Nedlands, Australia

*Corresponding author: Audrey A Margery-Muir, School of Biomedical Sciences, Curtin University, GPO box U1987 Bentley Western, Australia 6845, Tel: +08-926-687-903; E-mail: Audrey.muir@postgrad.curtin.edu.au

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Abstract

Systemic lupus erythematosus (SLE) is an inflammatory disorder in which autoantibodies contribute to impaired apoptosis and clearance of cell debris. Anti dsDNA and anti C1q antibodies have been implicated, as well as complement protein C1q itself. IgG autoantibodies reacting with the collagen-like region of C1q protein (α C1q ab) were quantitated in serum of 56 patients diagnosed with SLE and undergoing treatment for variable periods, together with 33 age/sex-matched controls. Analysis of the results showed optimal sensitivity and specificity of 57% and 91% respectively at a cut-off concentration for positivity of 20 U/ml. The assay is a potentially useful confirmatory test for SLE, but is not suitable as a screening test for SLE with the probability of a positive test and SLE in an individual within a random population of only $\leq 1\%$. α C1q ab concentrations were detectable in all samples tested with concentrations manifesting no correlation with age and serum C1q levels in SLE patients and a negative correlation with age in controls. The α C1q ab detected by this assay do not react therefore with native C1q. In SLE patients, α C1q ab concentrations correlated with the concentrations of dsDNA antibodies, ($p=0.0001$) and C- reactive protein and inversely with complement component C4 (C4) concentrations ($p=0.041$). α C1q ab concentrations were not associated with individual therapeutic regimens, but were higher in those patients receiving a combination of three drug therapies and with the presence of renal disease. The diagnostic relevance of this complex autoantibody will require further definition of its antigenic specificities.

Introduction

There is good evidence that impaired apoptosis and clearance of cell debris induces autoimmune responses associated with aberrant inflammation [1]. Systemic lupus erythematosus (SLE) is an important systemic autoimmune disease characterised by the presence of autoantibodies that bind double stranded DNA (henceforth dsDNA ab) and often decreased levels of serum complement components C3 and C4 [2]. Complement component C1q is one of three proteins comprising the first component (C1) of the classical pathway of complement activation. C1q molecules can activate the complement system by recognizing different structures on microorganisms and apoptotic bodies, or indirectly by means of binding to immunoglobulins and classical acute phase proteins such as C-reactive protein, CRP resulting in clearance of immune complexes and apoptotic material [2,3]. The avidity of binding of C1q to the Fc domain of IgG antibodies is augmented by the presence of hexameric IgG/antigen complexes on cell surfaces or polyvalent antigens [4]. A consequence of high affinity binding of C1q to Fc receptors is the formation of a neo-antigen on the collagen-like tails of the C1q molecule subunits [5]. Production of auto-antibodies reacting with this neo-antigen has been observed following C1q binding with immune complexes or nonspecific binding to chromatin [6]. The role of these autoantibodies in the pathogenesis of specific disease, for example SLE, is yet to be clearly defined [2]. Uwatoko and colleagues [7] devised an assay capable of quantifying anti-C1q antibodies (henceforth α C1q ab) by their interaction with C1q bound to a plastic surface in the presence of a high salt concentration (1M NaCl) to preclude any interaction with soluble C1q present in the test sample, usually serum.

In the last decade, the relationship between α C1q ab and clinical profiles in SLE patients, as well as relationships with other laboratory parameters has been investigated. It has been reported by several groups [6,8-10] that the levels of α C1q ab titers are usually significantly higher in SLE patients than healthy controls or non-SLE autoimmune patients. The concentrations of α C1q ab correlate with disease activity and/or severity [6,8-19] as well as with other laboratory parameters including complement protein C3 and/or C4 concentrations, dsDNA ab, proteinuria and the presence of immune complexes [6,8,11,12,14-19]. Whether serum concentration of α C1q ab is a useful tool to predict upcoming flares in patients with nephritis remains to be confirmed. A strong association between α C1q ab and active renal disease has been demonstrated [18]. Only nine studies have longitudinal data permitting this specific assessment (including 10,14), although other reports have supported its potential for this use [9,10,12,17]. The present study measured the levels of α C1q ab in a cohort of diagnosed and treated Western Australian SLE patients using a commercial ELISA kit.

Methods

Cohorts and ethics

Human ethics approval: Curtin University, Sir Charles Gairdner Hospital and Royal Perth Hospital Human Ethics Committees approved the human studies (approval numbers HR 202/2013, HREC 2013-174). Written and informed consent was obtained from all study participants.

Fifty-six (56) treated systemic lupus erythematosus patients aged 47 ± 15 years were recruited from Sir Charles Gairdner (SCGH) and

Royal Perth Hospitals (RPH-Perth, Western Australia) between March 2014 and December 2015. The diagnoses were based on the American College of Rheumatology (ACR) classification criteria. In addition to the SLE diagnosis, patients had co-morbidities including renal disease, liver pathologies as well as Sjögren and Raynaud's syndromes. These are summarised in (Table 1). SLE patients in this cohort were treated with various therapeutic regimens including steroids (low dose

prednisolone ($\leq 5\text{mg/day}$)), immunosuppressant therapies (azathioprine, cyclosporine, cyclophosphamide, methotrexate, mycophenolate, mofetil) and antimalarial therapies (hydroxychloroquine). Thirty-three age and sex-matched healthy control subjects (47 ± 15 years) were recruited from the Perth metropolitan area.

	Steroids (1)	Anti-malarial (2)	Immuno-suppressants (3)	1+2	1+3	2+3	Combination of 1, 2 & 3	Other therapeutics
Renal symptoms (N=11)	8 (72.7%)	7 (63.6%)	8 (72.70%)	1 (9.1%)	3 (27.3%)	0 (0%)	4 (36.4%)	8 (72.7%)
Hepatic involvement (N=4)	2 (50%)	4 (100%)	2 (50%)	1 (25%)	0 (0%)	1 (25%)	1 (25%)	3 (75%)
Anti-phospholipid (N=17)	13 (76.5%)	9 (52.9%)	7 (41.20%)	3 (17.60%)	3 (17.6%)	0 (0%)	4 (23.5%)	16 (94.1%)
Raynaud's phenomenon (N=4)	2 (50%)	2 (50%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	4 (100%)
Sjögren's disease (N=4)	0 (0%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (75%)
Cutaneous/discoid symptoms (N=12)	5 (41.7%)	6 (50%)	7 (58.30%)	1 (8.30%)	0 (0%)	0 (0%)	3 (25%)	9 (75%)
Neuronal symptoms (N=1)	1 (100%)	1 (100%)	0 (0%)	1(100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
T1DM (N=3)	3 (100%)	2 (66.7%)	2 (66.70%)	1(33.30%)	1 (33.3%)	0 (0%)	1 (133.3%)	3 (100%)
Other symptoms (N=13)	9 (69.2%)	11 (84.6%)	7(53.80%)	2(15.40%)	2 (15.4%)	1 (7.7%)	4 (30.8%)	13 (100%)

Table 1: The table indicates the number and percentage of patients in each category of lupus manifestation under specific types of treatment (Patients may fit into multiple lupus manifestations as well as treatments under review with other type of medications).

Sample collection

Peripheral blood from SLE patients was collected into ethylenediaminetetraacetic acid (EDTA), heparin and serum tubes at PathWest collection centres. Serum samples were separated at PathWest laboratories within one hour of collection using centrifugation and frozen to between -70°C and -80°C . Samples from healthy human controls were collected into EDTA, heparin and serum vacutainer tubes (Beckton Dickinson (BD), San Jose, USA) and processed in the same way at Curtin University laboratory (Perth, WA).

C1q ab concentrations

A solid-phase ELISA kit (Buhlmann Laboratories, Schönenbuch Switzerland) was used to quantify IgG αC1q ab. Stored patient sera were diluted in high salt buffer (0.5M NaCl) and incubated in microtiter wells coated with human C1q. Horseradish peroxidase (HRP) labeled anti-human IgG was then added, followed by tetramethylbenzidine (TMB) substrate. A washing step was included between each incubation. The substrate reaction was terminated through the addition 0.25M sulphuric acid and the absorbance measured at 450 nm using a microtiter plate reader (Bio-Rad). Test sample concentrations were interpolated using a four-parameter logistic calibration curve fitted to the standards provided by the manufacturer (5, 25, 100 and 400 U/mL based on an international reference standard). The manufacturer's suggested value for positivity was 15 units/ml (Figure 1).

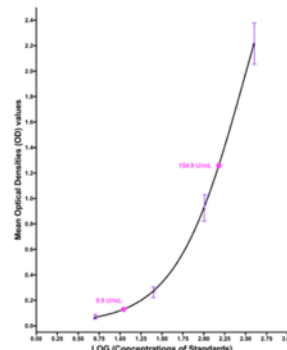


Figure 1: A four-parameter logistic standard curve for the αC1q ab kit (Buhlmann Laboratories) over the range of 5, 25, 100 and 400 U/ml (mean \pm standard deviation of optical density values).

Complement C1q, C3, C4 concentrations, CRP, double stranded DNA antibodies (dsDNA ab) assays

Patients data for dsDNA ab, CRP, C4 and C3 were collected from the patients' files and corresponded to the time-point at which they

were recruited and blood samples obtained for the study. All the above parameters were assayed in the PathWest Diagnostic Laboratories at Fiona Stanley Hospital, Fremantle and Sir Charles Gairdner Hospital. Samples tested at PathWest for antibodies to double stranded DNA: dsDNA ab were assayed using a radioimmunoassay dsDNA ab kit (Trinity Biotech; NY, USA) as per the manufacturer's directions. C-reactive protein (CRP) concentrations in serum or plasma samples were assessed using the turbidometric anti CRP antibody coated latex particle assay in an Abbott Architect C16000 instrument (Abbott Laboratories, Abbott Park, Illinois USA). Complement protein C3 and C4 concentrations in serum or plasma samples were also measured by turbidimetry assay using the Abbott Architect C16000 instrument. Complement C1q levels were assayed using a nephelometry method on a Siemens BN2 nephelometer.

Data analysis

Statistical significance was calculated using Mann-Whitney ranking U-test with the program GraphPad PRISM 7 (GraphPad Software Inc, California, USA). P values of <0.05 were considered statistically significant.

Results

Quantitation of anti-C1q antibody in treated SLE patients and controls

Multiple assays (including repeated samples) were performed in accordance with the manufacturers specifications using a range of international calibrator concentration standards (5, 25, 100 and 400 U/ml) provided with the kit. Two quality control samples provided by the manufacturer were included in each assay. These comprised a negative control with expected values between 4.0-7.9 U/mL and a positive control sample between 118-233 U/mL. The positive control was always within the expected concentration range. The negative control sample was marginally higher in our analysis (average of 9.9 U/mL) than that stipulated by Buhlmann Ltd, but still lower than the 20 U/mL cut-off point for positivity used in this analysis.

The non-Gaussian distributions of the concentration of the α C1q ab in patients and controls are shown in (Mann Whitney rank test analysis $p \leq 0.0001$) (Figures 2A-C).

The optimal specificity and sensitivity of the assay for inclusion or exclusion of SLE were estimated from the proportions of true positive and true negative results as a function of variable "cut-off concentrations of α C1q ab. From these data, a receiver-operator characteristic curve (ROC: plot of sensitivity (%) vs. 1-specificity (%), see Figure 3) was generated with an area under the curve of 0.78 (0.68 -0.88, 95% CI, $p=0.0001$).

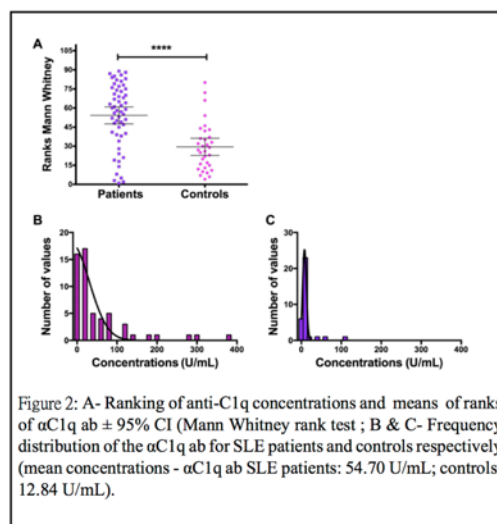


Figure 2: A- Ranking of anti-C1q concentrations and means of ranks of α C1q ab \pm 95% CI (Mann Whitney rank test ; B & C- Frequency distribution of the α C1q ab for SLE patients and controls respectively (mean concentrations - α C1q ab SLE patients: 54.70 U/mL; controls: 12.84 U/mL).

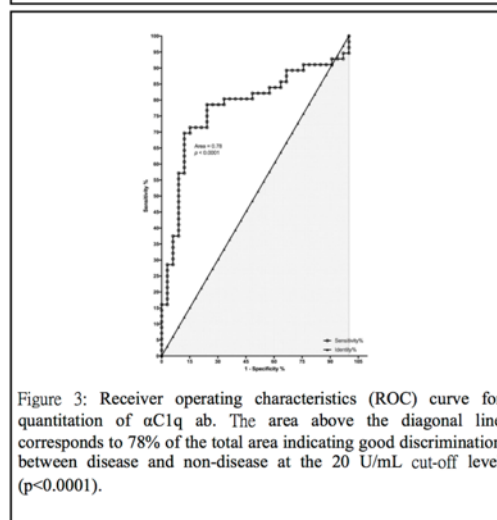


Figure 3: Receiver operating characteristics (ROC) curve for quantitation of α C1q ab. The area above the diagonal line corresponds to 78% of the total area indicating good discrimination between disease and non-disease at the 20 U/mL cut-off level ($p < 0.0001$).

From this graph the 20 U/mL cut-off concentration provided a likelihood ratio of 6.3 (i.e. probability of a positive test in patients with SLE/probability of a positive test in persons without SLE). This compares with the likelihood ratio of 5.0 for the 15 U/mL cut-off value recommended by the manufacturer, and often quoted by other groups using the same international control samples (6). Henceforth the 20 U/mL discrimination value has been used throughout this analysis. The sensitivity and specificity of the assay for the diagnosis of SLE were 57% and 91% respectively. Assuming an average prevalence of SLE of $\approx 0.1\%$ in the WA population [20], the estimated predictive value of a positive and negative α C1q ab test for SLE was 91% and 56%

respectively. Bayesian analysis results in the probability of a positive test and SLE in an individual within a random population of the order of 1-2%. There was a higher percentage of positive α C1q ab concentrations in SLE patients than in controls (57% vs. 9% respectively, ($p=0.0001$); Mann Whitney rank test), and it was noted that all patients and control samples tested manifested some level of α C1q ab.

Anti-C1q antibody concentrations correlate in SLE patients with dsDNA ab concentrations and inversely with complement C4 concentrations

Correlation coefficients were estimated for α C1q ab concentrations and those of several other serological analytes usually measured in SLE

testing. These are shown in Table 2. Significant correlations were observed between α C1q ab and dsDNA ab ($p=0.0001$); an inverse correlation between α C1q ab concentrations and complement component C4 concentrations was noted ($p=0.041$). The correlation matrix also revealed associations in SLE patients between C-Reactive Protein (CRP) and both dsDNA ab ($p=0.005$) and C1q complement protein levels ($p=0.024$). However, C1q protein levels in SLE patients were not significantly correlated with anti-dsDNA antibodies. There was a weak negative correlation (not statistically significant), between the age at time of testing of individual SLE patients and the concentration in serum of α C1q ab, whereas in the control cohort the concentration of α C1q ab was negatively correlated with age at time of collection ($p=0.001$).

	Age	(C4)	(C1q)	(C3)	(CRP)	(Ds-DNA ab)	(DNASE-I)	(Anti-C1q ab)
Age		-0.073	0.07	0.063	0.014	-0.275 (P=0.042)	0.034	-0.146
(C4)	-0.073		0.085	≤ 0.0001	0.203	-0.087	-0.045	-0.274 (P=0.041)
(C1q)	0.07	0.085		0.248	0.325 (P=0.024)	0.241	0.263 (P=0.050)	0.088
(C3)	0.006	0.116	-0.02		0.239	-0.118	0.017	-0.131
(CRP)						0.406 (P=0.005)	-0.063	0.136
(Ds-DNA ab)							0.059	($p \leq 0.0001$)
(DNASE I)	0.14	-0.141	0.23	0.225				0.038
(Anti-C1q ab)	-0.566 (P=0.001)	-0.246	-0.024	0.057			-0.368 (P=0.035)	

Table 2: Correlation matrix between α C1q ab and other serological parameters and age. The values above the diagonal are the Spearman (r) correlation values and when significant the significance levels in brackets ($p \leq 0.05$). The same comparisons, when available, are shown for the control population below the diagonal line.

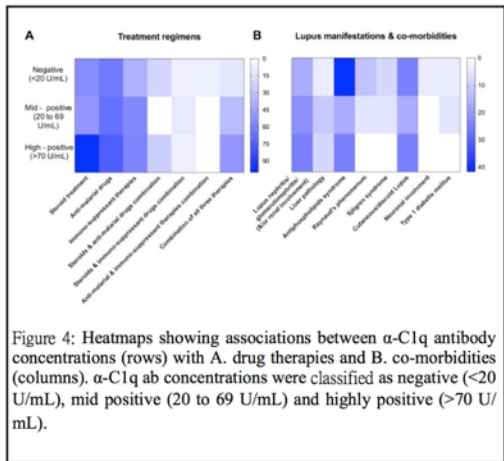


Figure 4: Heatmaps showing associations between α -C1q antibody concentrations (rows) with A. drug therapies and B. co-morbidities (columns). α -C1q ab concentrations were classified as negative (<20 U/mL), mid positive (20 to 69 U/mL) and highly positive (>70 U/mL).

Anti-C1q ab concentrations are associated with treatments and renal co-morbidities in patients

Heatmaps were used to show any patterns in the patient group linking concentrations of α C1q ab and therapeutic treatments, as well as co-morbidities. Figure 4A shows the association of the α C1q ab with the drug treatments offered to SLE patients over the course of their disease. It was observed that patients who received all three treatments of steroids, anti-malarial and immune-suppressants (7th lane) exhibited higher levels of C1q abs. Figure 4B shows associations of α C1q abs with the co-morbidities of the patients. The first lane shows an association of higher concentrations of α C1q abs in patients with diagnosed renal disease. In contrast, patients with hepatic involvement (3rd lane) exhibit lower levels of α C1q abs.

Discussion

C1q molecules can activate the complement system by recognizing different structures on microorganisms and apoptotic bodies, or indirectly by means of bound immunoglobulins and classical acute phase proteins such as C-reactive protein, (CRP) [3]. Following these processes the generation of a neo antigen on the α C1q ab concentrations were assayed in the serum of a cohort of SLE patients undergoing clinical management for varying durations, and a panel of 33 healthy controls using a commercially available ELISA (Buhlmann Laboratories). The ROC graph analysis of the test result data showed that 20 U/mL was a more discriminatory cut-off concentration than

the value of 15 U/mL recommended by the manufacturer and some other groups [8]. The relatively low sensitivity of the assay (57%) restricts the utility of the test for exclusion of SLE, however the high specificity of the assay (91%) provides a useful confirmatory test. Further, the probability of a positive test AND SLE in an individual in a random population is only of the order of 1-2%, thereby ruling out the assay as a screening test for SLE. It is noted that three of the 33 serum samples from the healthy control cohort exhibited high levels of α C1q ab; we cannot explain this anomaly. It is clear the presence of α C1q ab as detected by the ELISA assay used herein is not pathognomonic for SLE.

This study reports a point-in-time estimate of α C1q ab concentrations in the serum of a cohort of SLE patients under clinical management for varying durations. Hence, it is not possible in this study to relate α C1q ab levels to events of clinical significance such as flare of renal disease. α C1q ab levels were inversely correlated with serum C3 and C4 concentrations in SLE patients (albeit C3 not significantly) as would be expected if classical pathway complement activation was contributing to the disease process [8]. These observations may be confounded by treatments (such as immunosuppressant therapies) received by the SLE patients over the course of their disease. Both dsDNA ab and CRP levels were significantly positively correlated with α C1q ab concentrations suggesting that in SLE patients, some of these analytes contributed directly to the disease mechanism [2]. An important cognate study [21] demonstrated two human monoclonal anti-DNA antibodies (R4A and G11) that cross-reacted with a globular region (as opposed to the collagen-like region) antigenic determinant in mouse and human C1q proteins. Both antibodies bound both soluble C1q as well as solid phase C1q. Franchin et al., [21] postulated that anti-C1q antibodies could contribute to the development of immune complex mediated glomerulonephritis by direct binding to glomeruli or by removal of soluble C1q. In our study α C1q ab concentrations were not correlated with serum concentrations of C1q in either the patient or control cohorts. This implies that most of the α C1q ab detected in this assay are specific for non-globular antigenic epitopes on α C1q. The significant correlation of α C1q ab concentration with anti-dsDNA antibodies is consistent with the cross-reactivity reported by Franchin et al., [21], but may also just reflect two separate but related processes. It is also of interest that Vanhecke et al., [22] described α C1q ab reacting with peptide epitopes on the A and B collagen-like tails of human C1q that were not present on the C chain. These epitopes were only present on bound C1q and not on soluble C1q and mapped to different regions of the collagen-like region of C1q. This raises the possibility that the α C1q ab detected in this study may include a proportion of antibodies directed against the peptide epitopes described by Vanhecke [22]. It is clear the specificities of the α C1q ab observed in the serum of SLE patients need to be characterised in more detail, especially if different antibody specificities reflect distinct attributes of SLE pathology eg presence of nephritis. Further, as C1q is synthesised mainly by macrophages and immature dendritic cells, serum C1q levels may be relatively insensitive to C1q consumption within tissues [23,24].

During a primary immune response C1q is activated by binding to pentavalent Fc structures in IgM antibodies. In secondary immune responses, high avidity binding and activation of C1q is achieved by binding to Fc domains of antigen bound IgG molecules associated in hexameric clusters located in membranes of cells [4]. In both instances, it is known that a neo-antigen(s) is generated on the C1q molecule that elicits synthesis of α C1q ab [25]. Thus, it would be expected that the

sequelae of humoral immune responses to diverse antigens may result in neo C1q antigen formation and subsequent autoantibody synthesis. Clearly, these autoantibodies would not be expected to be specific for SLE and hence constitute a more general epiphenomenon. This statement is consistent with the presence of α C1q ab reported in the serum of individuals with a variety of non-SLE autoimmune disorders and infectious diseases, as well as in a proportion of healthy individuals (reviewed by [5]). The enigmatic role of C1q in SLE is emphasized further by the recent review (by international survey) of C1q deficiency in humans [26] in which the authors reported a high prevalence ($\approx 80\%$) of SLE, together with decreased survival times and increased susceptibility to infections. Furthermore, C1q has been linked to activation of a tumour suppressor locus with prostatic hyperplasia and carcinoma [27].

It seems there are at least two distinct mechanisms in the predisposition to SLE explained by C1q. SLE occurs with high frequency in C1q protein deficient persons [26,28] and SLE can also occur in the presence of normal C1q protein levels and relatively high concentrations of α C1q ab that contribute to inflammatory processes [16]. SLE patients who received all three classes of drug therapies tended to have higher levels of α C1q ab. This suggests that patients with a more severe disease phenotype requiring multiple therapeutic treatments to reduce organ damage also produce higher levels of α C1q ab. In contrast, patients with less severe disease, requiring fewer therapeutic agents and manifesting lower levels of α C1q ab. This observation supports, but does not confirm, reports that relate high α C1q ab levels to renal symptoms and/or renal flares [18]. There have been many studies over the past two decades of α C1q ab levels in disease, including SLE [17,18]. These, together with this report, suggest that quantitation and standardization of anti-C1q antibodies has a useful role in the classification of subtypes of SLE and as a potential confirmatory diagnostic and monitoring assay [17,18].

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B.2.3 Genetic Variation at the DNASE I Locus in an Australian Cohort of SLE Patients (Lupus: Open Access)



Lupus: Open Access

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Research Article

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Genetic Variation at the *DNASE I* Locus in an Australian Cohort of SLE Patients

Audrey A Margery-Muir*, John D Wetherall and David M Groth

School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, GPO box U1987 Bentley, Australia

Abstract

Objective: *DNASE I* serum concentrations and activity, as well as sequence mutations have been implicated in the pathology of systemic lupus erythematosus (SLE). This study was undertaken to assess the serum *DNASE I* concentrations and assess the *DNASE I* sequence variation in a cohort of SLE patients and controls.

Methods: *DNASE I* serum concentrations were assayed in 56 SLE patients and 33 age and sex matched controls. All SLE patients and controls were genotyped for exomic alleles at the *DNASE I* locus and for the variable number tandem repeat alleles present in intron 4 (VNTR - HumDN1).

Results: Skewed *DNASE I* protein concentration distributions were observed with the mean value for SLE patients being 44.2 U/mL compared to 56.4 U/mL in controls (NS). No sample tested negative for *DNASE I* protein. Only two of the previously reported six exomic alleles (*DNASE**1, *DNASE**2) were identified, together with four VNTR alleles (three-six repeats). Both loci manifested Hardy-Weinberg equilibrium. Linkage disequilibrium was observed between exomic alleles and the VNTR alleles, especially between *DNASE**1 and 4 repeat VNTR (HumDN1) allele. No significant associations were observed between *DNASE I* concentrations and genotypes. Estimations of haplotype frequencies showed similar distributions for both SLE and the control cohorts, although it was noted that haplotypes containing *DNASE**2 had an elevated frequency of longer VNTR alleles than did *DNASE**1.

A meta-study of *DNASE I* exomic allelic frequencies showed similar frequencies to those obtained in other populations. For the VNTR locus, the longer alleles (five and six repeats) were more frequent, although there was no difference between SLE patients and controls.

Conclusion: This study does not support the hypothesis that specific two locus *DNASE I* genotypes predispose to SLE in the Western Australian cohort.

Keywords: Systemic lupus erythematosus; Serum *DNASE*; Autoimmune disease

Introduction

Systemic lupus erythematosus (SLE) is an important systemic autoimmune disease characterised by the presence of autoantibodies binding double stranded DNA (henceforth dsDNA ab) and decreased levels of serum complement components C3 and C4 [1]. It has been postulated that defective apoptotic clearance of immune complexes incorporating histone associated DNA predisposes to SLE [1]. *DNASE I*, is an endonuclease [2] believed to facilitate the removal of cellular debris thereby preventing aberrant inflammation [3-5]. There have been reports that *DNASE I* deficiency is a predisposing factor for SLE. Lower levels of *DNASE I* enzyme activity may be associated with lupus nephritis [6,7] and correlate with disease activity state and clinical parameters [8]. Yasuda and colleagues [2,9-12] and Iida et al. [13] have identified exomic polymorphisms of the *DNASE I* gene, some of which appear to be associated with susceptibility to SLE [14]. Furthermore, Koji and colleagues [15] have described an A-G transversion in exon

two that results in defective synthesis of *DNASE I* in an SLE patient manifesting lower *DNASE I* activity. The *DNASE I* gene also contains a polymorphic 56bp variable number tandem repeat (VNTR-HumDN1) locus within intron four manifesting six alleles corresponding to molecular product sizes of 469, 525, 581, 637 and 693 for two, three, four, five and six repeats respectively [16-18]. Yasuda and colleagues [16] reported that the three repeats allele of VNTR (HumDN1) was in linkage disequilibrium with *DNASE I**1 allele whilst the VNTR alleles four and five were in linkage disequilibrium with *DNASE I**2.

In this study, genetic polymorphisms at both the *DNASE I* locus and at the VNTR (HumDN1) locus were typed in a Western Australian

population of SLE patients and controls with known serum concentrations of *DNASE I* protein. The genetic diversity of *DNASE I* polymorphisms in these cohorts was compared with other studies and associations sought with disease and serological parameters indicative of SLE pathology.

Methods

Patients, controls and ethics

Fifty-six (56) treated systemic lupus erythematosus patients aged 47 ± 15 yrs. were recruited between March 2014 and December 2015 from Sir Charles Gairdner (SCGH) and Royal Perth Hospitals (RPH-Perth, Western Australia). The diagnoses were based upon the American College of Rheumatology (ACR) classification criteria. In addition to the SLE diagnosis, patients had co-morbidities including renal disease, liver pathologies as well as Sjögren and Raynaud's syndromes. SLE patients in this cohort were treated with various therapeutic regimens including steroids (low dose prednisolone (≤ 5 mg/day)), immunosuppressant therapies (azathioprine, cyclosporine, cyclophosphamide, methotrexate, mycophenolate, mofetil) and

population of SLE patients and controls with known serum

antimalarial therapies (hydroxychloroquine). Thirty-three age and sex-matched healthy control subjects (47 ± 15 yrs.) were recruited from the Perth metropolitan area. Human ethics approval: Curtin University, Sir Charles Gairdner Hospital and Royal Perth Hospital Human Ethics Committees approved the human studies (approval numbers HR 202/2013, HREC 2013-174). Written and informed consent was obtained from all study participants.

Sample collection

Peripheral blood from SLE patients was collected at one time point during the course of their disease monitoring and management into ethylenediaminetetraacetic acid (EDTA), heparin and serum tubes at PathWest collection centres. Serum samples were separated at PathWest laboratories within one hour of collection using centrifugation and frozen to between -70°C and -80°C . Samples from healthy human controls were also collected into EDTA, heparin and serum vacutainer tubes (Beckton Dickinson (BD), San Jose, USA) and processed in the same fashion at the Curtin University laboratory (Perth, WA).

Identification of phenotypes and genotypes at the *DNASE I* and intron 4 VNTR (HumDN1) loci

Stored DNA samples were used in a PCR reaction using MyFi™ DNA polymerase (Bioline, USA) to amplify exons (or part of) two, five, six, seven and eight using primers sets (from GeneWorks, Australia-table S4). The *DNASE I* gene was detected by PCR amplification using primers as indicated in Supplementary Table S4 in PCR microfuge tubes containing 1 μL of test DNA, together with 5 μL of 5x MyFi reaction buffer (Bioline, USA), 0.5 L of each forward and reverse primers 10 μM and 1 μL of MyFi DNA polymerase (Bioline, USA) as well as 17 μL of nuclease free water for a total volume of 25 μL per reaction. PCR assays were performed with cycle parameters set at 95°C for 1 min (1 cycle) and 35 cycles consisting of 95°C for 15 s followed by 61°C for 15 s (*DNASE I*) or 57°C for 15 s (VNTR-HumDN1).

PCR products were then sequenced (MACROGEN, Seoul, Korea) to identify exonic alleles of Human *DNASE I* as indicated in Table 1.

The number of VNTR (HumDN1) repeats within intron 4 of the Human *DNASE I* was determined using a PCR reaction with MyFi™ DNA polymerase (Bioline, USA) to amplify intron four spanned by exons four and five using the primers pair shown in Supplementary Table S4 (GeneWorks, Australia). The lengths of the amplicons were then estimated on a 4% agarose gel electrophoresis using a 1kb DNA ladder. As a control measure, five samples were sent for sequencing (details as above) and the results compared with the gels. Alleles manifested three, four, five and six repeats with corresponding molecular weights of 525, 581, 637 and 693bp respectively [19].

DNASE I enzyme concentrations

A sandwich ELISA kit (Creative Diagnostics, USA) was used to assay human *DNASE I* serum concentrations per manufacturer's instructions. Briefly, stored patient sera were incubated in microtiter wells coated with a *DNASE I* specific antibody. *DNASE I* present in

the samples was immobilized and unbound products removed. Biotin labelled *DNASE I* specific antibody bound to immobilized *DNASE I* was detected with avidin conjugated Horseradish Peroxidase (HRP). Test sample concentrations were interpolated using a four-parameter logistic calibration curve fitted to the standard sera provided by the manufacturer (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0 ng/mL).

Other serological parameters associated with SLE

We have previously reported the methods for assaying C1q, C3, and C4 concentrations in the serum of the SLE and control cohorts [20].

Data analysis

Nucleotide and peptide sequences were analysed using Geneious version 10 [21]. Linkage disequilibrium and population parameters were determined using "Genepop on the web" [22,23], LinkDos [24] and SNPStats [25]. Statistical significance was calculated using Mann-Whitney rank sum U-test with the GraphPad PRISM 7 application (GraphPad Software Inc., California, USA). P values of <0.05 were considered statistically significant.

Results

DNASE I protein concentrations in SLE patients and controls

DNASE I protein concentrations observed in serum samples of SLE patients and controls are shown in Figure 1. The distribution of concentrations in both cohorts was skewed; the mean concentration of *DNASE I* protein in SLE patients was lower than that of the controls (44.2 U/mL vs. 56.4 U/mL) although the difference was not significant. A preliminary investigation of associations between *DNASE I* serum concentrations and co-morbidities and drug therapies in the SLE patients was undertaken using heat maps is shown in Figures 2C and 2D respectively.

Allele and genotype frequencies at the *DNASE I* and intron 4 VNTR (HumDN1) loci

Allele frequencies at the *DNASE I* exonic and VNTR (HumDN1) loci are summarised in Table 2.

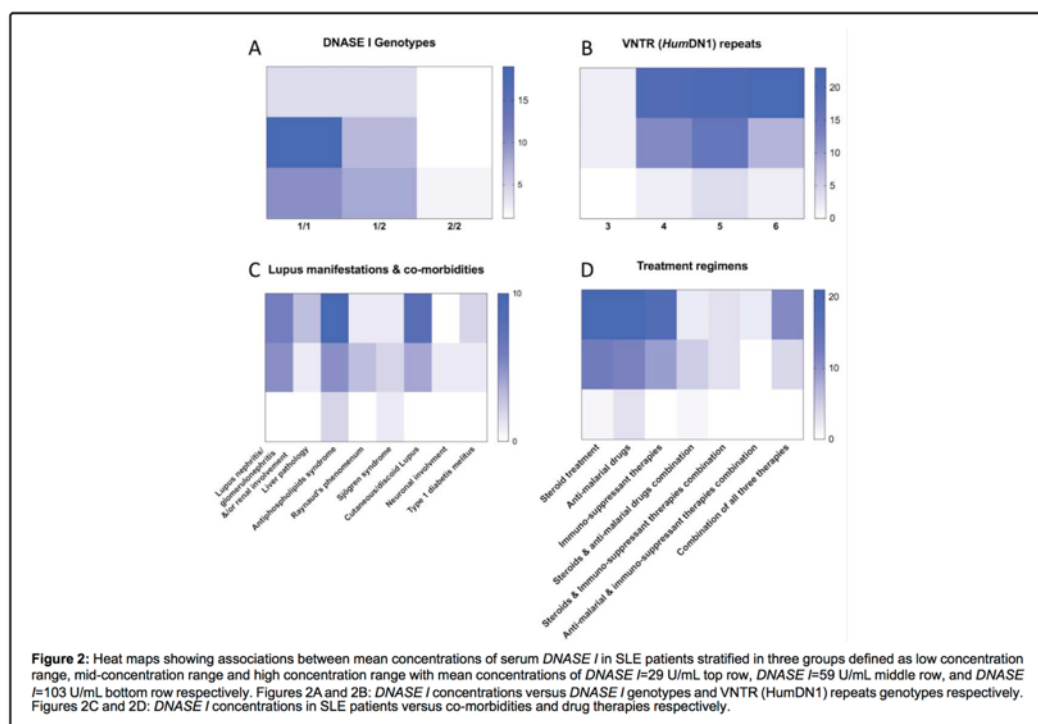
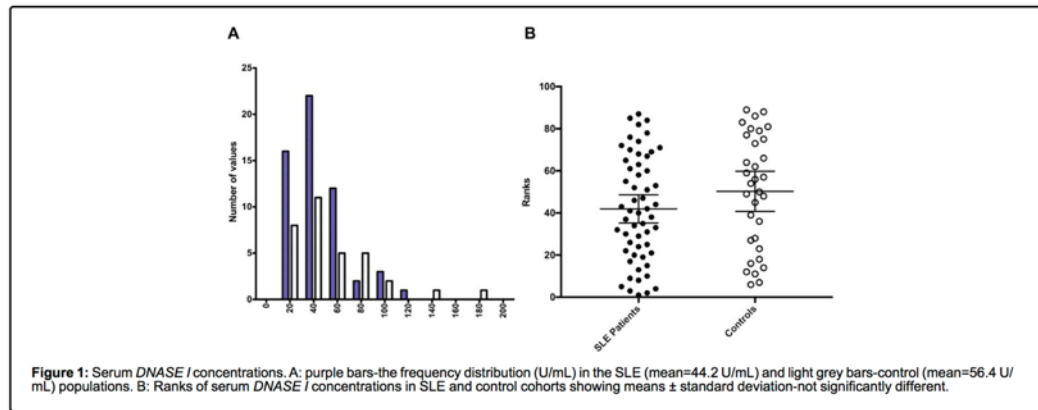
Alleles at both intragenic loci exhibited Hardy-Weinberg equilibrium and linkage disequilibrium. The frequencies of haplotypic combinations at both the *DNASE I* and VNTR (HumDN1) loci are shown in Figure 3. Haplotypes with VNTR alleles of 4, 5 and 6 repeats were more prevalent in both the SLE and control cohorts.

Ethnic variation in *DNASE I* polymorphisms

The allele frequencies reported in this study are in general agreement with the metastudy of ethnic allele frequency variation shown (see Table 3). Only the Ovambos' of African origin have higher frequencies of the *DNASE I* P*1 relative to the *DNASE I* P*2 allele. No apparent significant differences between *DNASE I* allele frequencies were observed for either the SLE patients or the healthy controls. A comparison of ethnic variation in the frequencies of the internal VNTR (HumDN1) locus within the different

<i>DNASE I</i> allele	Exon 2	Exon 5	Exon 6	Exon 7	Exon 8
<i>DNASE I</i> P*1	CAG-Gln	GTG-Val	CCC-Pro	CGC-Arg	CAA-Gln
<i>DNASE I</i> P*2	CAG-Gln	GTG-Val	CCC-Pro	CGC-Arg	CGA-Arg
<i>DNASE I</i> P*3	CAG-Gln	GTG-Val	GCC-Ala	CGC-Arg	CAA-Gln
<i>DNASE I</i> P*4	GAG-Glu	GTG-Val	CCC-Pro	CGC-Arg	CGA-Arg
<i>DNASE I</i> P*5	CAG-Gln	ATG-Met	CCC-Pro	CGC-Arg	CGA-Arg
<i>DNASE I</i> P*6	CAG-Gln	GTG-Val	CCC-Pro	TGC-Cys	CGA-Arg

Table 1: PCR products sequenced to identify exonic alleles of Human *DNASE I*.



Population	<i>DNASE I</i> P1	<i>DNASE I</i> P2	VNTR (HumDN1) 3	VNTR (HumDN1) 4	VNTR (HumDN1) 5	VNTR (HumDN1) 6
SLE (n=56)	0.393 (n=44)	0.607 (n=68)	0.036 (n=4)	0.304 (n=34)	0.366 (n=41)	0.295 (n=33)
Control (n=33)	0.394 (n=26)	0.606 (n=40)	0.000	0.409 (n=27)	0.348 (n=23)	0.242 (n=16)

Table 2: Allele frequencies for the *DNASE I* locus and the intron 4 VNTR (HumDN1) locus in Australian SLE patients and healthy controls (numbers in brackets indicate the number of alleles in each group).

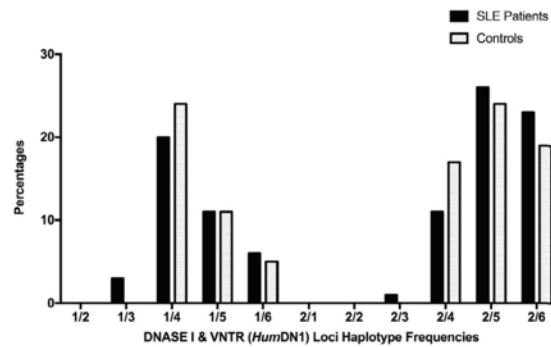


Figure 3: DNASE I (left number) & VNTR (HumDN1) (right number) Loci Haplotypes. The figure shows percentage haplotype frequencies in both loci for the SLE patients and control cohort.

Population (References)	Allele DNASE I*1 Not diseased	Allele DNASE I*2 Not diseased	Allele DNASE I*1 SLE	Allele DNASE I*2 SLE
Mixed Europeans (this study) Controls (n=25) (SLE n=24)	0.38	0.62	0.375	0.625
Mixed Asians (this study) Controls (n=5) SLE (n=11)	0.5	0.5	0.454	0.545
Mixed other (this study) Controls (n=3) SLE (n=21)	0.333	0.667	0.381	0.619
Germans [26,27]	0.294	0.705		
Germans [28]	0.265	0.728		
Spanish [29]	0.3003	0.6997	0.2246	0.7754
Japanese [26]	0.547	0.445		
Japanese [28]	0.595	0.405		
Koreans [28]	0.597	0.403		
Chinese (Han) [30]	0.536	0.464		
Chinese (Shenyang) [31]	0.567	0.433		
Chinese (Guangzhou) [31]	0.5417	0.4583		
Argentineans [14]	0.28	0.72	0.34	0.66
Turks [18]	0.221	0.779		
Ovambos [18]	0.872	0.128		
Ovambos [32]	0.872	0.128		
Ovambos [28]	0.889	0.111		
Indian Tamils [33]	0.52	0.49	0.495	0.505

Table 3: Frequency data for the DNASE alleles *1 and *2 are derived from published reports. The rare DNASE I*3, *4, *5 and *6 alleles are not included due to insufficient published data. "Mixed other" control group contained individuals of Middle Eastern and Indian ethnicity whereas the "Mixed other SLE" group included individuals from African, South American and Australian Indigenous ethnicities which were grouped together as there were only a small number of individuals in each.

ethnic groups is shown in Table 4. The frequencies of the six alleles reported in this study were similar to those observed in other population with the exception that the long six repeat allele was more frequent in both the control and SLE populations reported herein. The genetic differentiation of the Ovambo and Japanese populations was evident with the three-repeat allele being more prevalent than in the other populations reported.

Are serum DNASEI concentrations dependent on genotype?

DNASE I serum concentration distributions are shown ranked by genotype in Figure 4.

There were no significant associations between DNASE I serum concentrations and either the DNASE I exonic genotypes or the repeat number variation of the VNTR (HumDN1) locus. The lowest concentration levels of serum DNASE I were associated with DNASE I*2 genotype, and with the higher number of repeats at the VNTR (HumDN1) locus.

Associations between serological parameters and DNASE I and VNTR (HumDN1) alleles

A correlation matrix was generated for age, ethnicity, [C1q], [C3], [C4] and [DNASE I] versus VNTR (HumDN1) and DNASE I alleles (Supplementary Table S1). No significant correlations between the various parameters were observed except for serum concentrations of DNASE I and C1q complement protein ($r=0.653$; $p=0.05$). In the SLE cohort, the VNTR (HumDN1) repeat number was significantly correlated with [C3] and [C4] (0.304 ; $p=0.03$ and 0.279 ; $p=0.04$ respectively). However, specific alleles at both the VNTR (HumDN1) and DNASE I loci were significantly correlated in the SLE and control cohorts ($r=0.760$; $p=0.0001$; 0.569 ; $p=0.0005$ respectively) (Supplementary Tables S2 and S3).

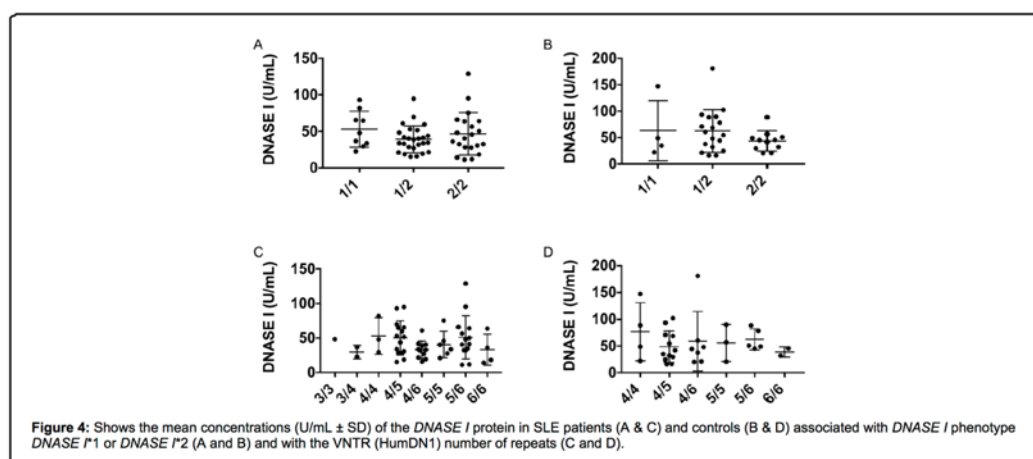
In the absence of well-defined associations between specific DNASE I genotypes and SLE, heat maps were used to visualize trends in disease severity that may be related to DNASE I genotypes or serum concentrations in both the SLE patient and control cohorts. The heat map analyses (Figure 2) show qualitatively that SLE patients with higher numbers of VNTR (HumDN1) repeats (≥ 5) had lower serum concentrations of DNASE I protein and required more intensive drug regimens for management of their disease than persons with low numbers of repeat motifs.

Discussion

In this investigation only the two most frequent (i.e. DNASE I*1 and DNASE I*2) of the six exonic alleles defined at the DNASE I locus were observed, although the typing system (shotgun sequencing) would have detected them if present. No variations in the nucleic acid sequence within exons two, five and seven were present in any of the individuals tested. The remaining four alleles (DNASE I*3, *4, *5 and *6) are rare and there are few reports of their frequencies in the other populations [26]. The frequencies of the DNASE I*1 and *2 in persons of European and Asian ethnicity were similar to previously published reports [26-

Population (References)	Allele 1 Not diseased	Allele 2 Not diseased	Allele 3 Not diseased	Allele 4 Not diseased	Allele 5 Not diseased	Allele 6 Not diseased	Allele 1 SLE	Allele 2 SLE	Allele 3 SLE	Allele 4 SLE	Allele 5 SLE	Allele 6 SLE
Mixed Europeans (this study) controls (n=50) SLE (n=48)	0	0	0	0.38	0.34	0.28	0	0	0	0.25	0.44	0.31
Mixed Asians (this study) controls (n=10) SLE (n=22)	0	0	0	0.6	0.4	0	0	0	0.05	0.41	0.36	0.18
Mixed other (this study) controls (n=6) SLE (n=42)	0	0	0	0.17	0.5	0.33	0	0	0.07	0.31	0.29	0.33
German [16]	0	0.01	0.26	0.34	0.39	0.01						
German [34]	0	0	0.39	0.33	0.28	0						
Japanese [16]	0	0.01	0.56	0.27	0.16	0						
Japanese [35]	0	0.03	0.58	0.21	0.18	0						
Chinese [35]	0	0.02	0.47	0.3	0.21	0						
Turks [35]	0	0	0.24	0.37	0.4	0						
Kuwaiti [17]	0	0.01	0.33	0.39	0.16	0.1	0	0.03	0.27	0.26	0.45	0
Kuwaiti [19]	0	0.02	0.31	0.37	0.18	0.12	0	0.05	0.28	0.26	0.42	0
Ovambo [35]	0	0.09	0.79	0.12	0.01	0						
Ghanaian [34]	0.01	0.08	0.77	0.09	0.05	0						
Iranian [36]	0	0	0.32	0.41	0.21	0.06	0	0	0.25	0.41	0.28	0.06

Table 4: Frequency data for the VNTR (humDN1) number of repeats are derived from referenced published reports. The rare *DNASE I* 3; *4; *5 and *6 alleles are not included due to insufficient data. "Mixed other" control group contained individuals of Middle Eastern and Indian ethnicity whereas the "Mixed other SLE" group included individuals from African, South American and Australian Indigenous ethnicities which were grouped together as there were only a small number of individuals in each.



28]. Four alleles were observed at the VNTR locus with the number of repeats varying from three to six. A greater frequency of the longest six repeat allele was observed in both cohorts in this study which may reflect improved typing techniques. Only in the Ovambo and Ghanaian populations did allele frequencies at both loci differ markedly from the values reported in other populations. This is consistent with an ancestral version of the *DNASE I* gene being present in modern day African populations [18].

Allele frequencies at both the *DNASE I* and VNTR (HumDN1) loci manifested Hardy-Weinberg equilibrium despite the relatively small sample size of the populations typed. As would be expected, both intragenic loci exhibited linkage disequilibrium (LD) as has been previously reported [16,37].

Of the 36 possible genotypes comprising observed alleles at these two loci, 13 were observed in the SLE patients and 12 in the control

group. These data demonstrate the presence of a restricted number of haplotypes in our populations. Al Fadhli and co-workers have shown that higher number of the VNTR (HumDN1) repeats are a risk factor for SLE and that the allele with five repeats was associated with a reduction of *DNASE I* enzyme activity [17]. It was not possible to measure the *DNASE I* enzyme activity in this study, however serum protein *DNASE I* concentrations were assayed in both populations. All persons tested exhibited *DNASE I* protein in their serum. Significant differences in serum *DNASE I* protein concentrations between SLE patients and the control cohort were not observed, although the mean concentration in the SLE cohort was slightly lower than in the control group. If *DNASE I* activity is proportional to the concentration of *DNASE I* protein it is unlikely that non-expressed mutant genes were present in the cohorts investigated.

Associations between the VNTR (HumDN1) locus and complement C3 and C4 serum concentrations were observed in the SLE cohort but

not in the control group. Hence, the number of VNTR (HumDN1) repeats in the SLE patients may influence clinical parameters. AlFadhli and co-workers have shown that higher number of the VNTR (HumDN1) repeats are a risk factor for SLE, and moreover, that the five-repeat allele was associated with a reduction of *DNASE I* enzyme activity [17].

The heat map analysis (Figure 2) showed qualitatively that SLE patients with higher numbers of VNTR (HumDN1) repeats (≥ 5) had lower serum concentrations of *DNASE I* protein than the controls and required more intensive drug regimens for management of their disease. However, quantitative analysis did not reveal any significant correlations between the serum concentrations of *DNASE I* protein and either phenotypes at the *DNASE I* and VNTR (HumDN1) loci or with disease severity (Figure 4). Other reports [17,36] in particular that of AlFadhli [17] show that a high number of VNTR repeats are a risk factor for SLE and may cause decreased *DNASE I* activity. Positive correlations between *DNASE I* and C1q serum concentrations and the length of the VNTR allele with serum concentrations of complement components C3 and C4 were observed and noted. If low *DNASE I* activity and/or concentration impairs removal of apoptotic material thereby predisposing to SLE, it might be expected that VNTR alleles negatively correlate with C3, C4 and C1q serum concentrations in SLE patients. Hence this report does not support the hypothesis that specific two locus *DNASE I* genotypes predispose to SLE in the Western Australian cohort.

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Appendix C Authors authorisation forms

C.1 Gender Balance in patients with systemic lupus erythematosus

To whom it may concern,

I, Audrey Margery-Muir, as the first author of the publication entitled "Gender balance in patients with systemic lupus erythematosus", Autoimmunity Reviews (2017) 258 – 268, declare that this work was primarily designed, interpreted, and written by me.


First author signature

I, as a co-author, endorse that this level of contribution by the first author indicated above is appropriate.

Christine Bundell

Co-author one printed name

Delia Nelson

Co-author two printed name

David M. Groth

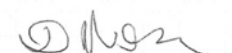
Co-author three printed name

John D. Wetherall

Co-author four printed name

Christine S. Bundell | Digitally signed by Christine S. Bundell
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Co-author three signature


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C.2 Anti-C1q Antibodies Concentrations by Elisa in Systemic Lupus erythematosus

To whom it may concern,

I, Audrey Margery-Muir, as the first author of the publication entitled "Anti-C1q antibodies concentrations by Elisa in systemic lupus erythematosus", Lupus: Open Access (2017) 2:1, declare that this work was primarily designed, experimentally executed, interpreted and written by me.



First author signature

I, as a co-author, endorse that this level of contribution by the first author indicated above is appropriate.

John D. Wetherall

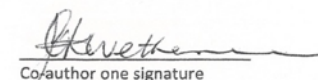
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David M. Groth

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Christine Bundell

Co-author three printed name



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Co-author two signature

Christine S. Bundell

Co-author three signature

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C.3 Insights on the relationship between complement component C4 serum concentrations and C4 gene copy numbers in a Western Australian systemic lupus erythematosus cohort

To whom it may concern,

I, Audrey Margery-Muir, as the first author of the manuscript entitled "Insights on the relationship between complement component C4 serum concentrations and C4 gene copy numbers in a Western Australian systemic lupus erythematosus cohort", currently submitted and under revision for publication with the Journal of Clinical Immunology (Springer), declare that this work was primarily designed, interpreted, and written by me.

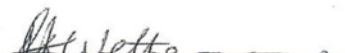

First author signature

I, as a co-author, endorse that this level of contribution by the first author indicated above is appropriate.

Christine Bundell
Co-author one printed name


Co-author one signature

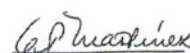
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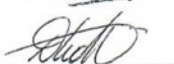
Rebecca Whidborne
Co-author three printed name


Co-author three signature

Patricia Martinez
Co-author four printed name


Co-author four signature

David M. Groth
Co-author five printed name


Co-author five signature

C.4 Genetic Variation at the DNASE I Locus in an Australian Cohort of SLE Patients

To whom it may concern,

I, Audrey Margery-Muir, as the first author of the publication entitled "Genetic Variation at the DNASE I Locus in an Australian Cohort of SLE Patients", *Lupus: Open Access* (2017) 2:3, declare that this work was primarily designed, experimentally executed, interpreted and written by me.



First author signature

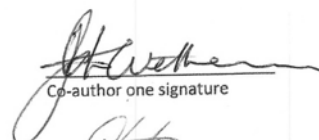
I, as a co-author, endorse that this level of contribution by the first author indicated above is appropriate.

John D. Wetherall

Co-author one printed name

David M. Groth

Co-author two printed name



Co-author one signature



Co-author two signature